Doctoral Program of Integrative Life Sciences
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Department of Veterinary Biosciences, Faculty of Veterinary Medicine
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# It's complex: studies on the genetics of canine hip dysplasia

Lea Mikkola

DOCTORAL DISSERTATION

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**Supervisors**

Professor Antti Iivanainen, DVM, PhD
University of Helsinki
Helsinki, Finland

Professor Hannes Lohi, PhD
University of Helsinki
The Folkhälsan Institute of Genetics
Helsinki, Finland

Docent Anu Lappalainen, DVM, PhD
University of Helsinki
Helsinki, Finland

**Reviewers**

Professor Rory J. Todhunter, DVM, PhD
University of Cornell
New York, United States

Professor Bart Broeckx, DVM, PhD
University of Ghent
Merelbeke, Belgium

**Opponent**

Jeffrey Schoenebeck, PhD
University of Edinburgh
Edinburgh, United Kingdom

Cover image: Milla Salonen

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# Abstract

This thesis addresses the genetic background of hip dysplasia, a complex hereditary disorder common in many species, including dogs and humans. The highly polygenic nature of hip dysplasia has become evident after many years of arduous research conducted in numerous studies that have explained only a small proportion of the disease heritability in dogs. The studies included in this thesis revealed multiple new loci, a novel regulatory variant for a gene that is imperative to normal joint development and validated numerous loci that have been associated with the disorder.

In **Study I**, genome-wide association analyses uncovered four loci that associated with the disorder with either protective or risk effects. A subsequent targeted resequencing and variant analysis found disease associated variants for multiple genes. Deletion variants in the putative regulatory region of the gene *NOG* were found to protect against moderate-to-severe hip dysplasia. These variants were shown to downregulate reporter gene expression *in vitro*.

In **Study II**, different hip dysplasia and osteoarthritis phenotypes were investigated. The genome-wide association analyses revealed three novel loci associated with hip joint incongruity and osteoarthritis. New candidate genes were identified: *NOG* and *NANOS1* for hip joint incongruity and *NOX3* and *ARID1B* for osteoarthritis.

In **Study III**, a total of 21 loci on 14 different chromosomes were validated in across- and within-breed association analyses in a large cohort of dogs comprised of 10 breeds. One locus was specific to the across-breed data. Neddylation-pathway was found to be significantly enriched in a candidate gene set that included 254 genes from the 21 validated loci.

These studies have generated new, long-awaited knowledge about the complex genetic background of hip dysplasia and related osteoarthritis in dogs. They also highlighted that mild hip dysplasia and the more severe disease forms may be induced by partially different genetic factors. Validation of associated loci for hip dysplasia has not been conducted to this extent before these studies, so an important step forward was taken in our latest study. Finally, finding causal variants has been uncommon. The regulatory *NOG* variants were an exciting finding, although their causality in hip dysplasia is yet to be revealed. Overall, our studies emphasise the complexity of the genetic background of hip dysplasia.

# Tiivistelmä

Tässä väitöskirjatutkimuksessa selvitettiin lonkkaniveldysplasian geneettistä taustaa. Lonkkaniveldysplasia on yleinen monitekijäinen sairaus, jota esiintyy kaikilla nisäkkäillä, kuten koirilla ja ihmisillä. Tämän sairauden perinnöllistä taustaa on selvitetty monissa eri tutkimuksessa lähes kahden vuosikymmenen ajan, mutta tulokset ovat selittäneet vain pienen osan sairauden periytymisasteesta. Tutkimuksemme toi esiin useita uusia perimän alueita, jotka liittyvät sairauteen. Löysimme myös uuden perimän muutoksen, joka sijaistee nivelten normaalille kehitykselle oleellisen geenin mahdollisella säätelyalueella. Lisäksi vahvistimme useita aiemmin julkaistuja perimän alueita.

Ensimmäisessä osatyössä löysimme neljä perimän aluetta kahdessa eri kromosomissa, jotka liittyivät lonkkaniveldysplasiaan, ja joilla oli joko lonkkaniveldysplasialta suojaavia tai sairastumisriskiä lisääviä vaikutuksia. Yhden tällaisen alueen kohdennettu sekvensointi ja varianttianalyysi paljasti useita muutoksia koirien perimässä, jotka liittyivät lonkkaniveldysplasian ilmiasuun. *NOG*-geenin mahdollisella säätelyalueella olevat 24-27 emäksen häviämien havaittiin suojaavan koiria vakavammilta lonkkaniveldysplasian muodoilta. Osoitimme, että nämä häviämät vähensivät reportterigeenin ekspressiota *in vitro*.

Toisessa osatyössä tutkimme erilaisia lonkkaniveldysplasiaa kuvaavia ilmiasuja sekä nivelrikkoa. Perimänlaajuinen assosiaatioanalyysi paljasti kolme perimän aluetta kolmesta eri kromosomista, jotka liittyivät eri lonkkanivelen epäyhdenmukaisuutta eli inkongruenssia kuvaavaan ilmiasuun sekä nivelrikkoon. Löysimme näiltä perimän alueilta useita kandidaattigeenejä. Geenit *NOG* ja *NANOS1* sijaitsivat lonkan inkongruenssiin liittyvällä alueella, kun taas geenit *NOX3* ja *ARID1B* olivat nivelrikkoon liittyvällä perimän alueella.

Kolmannessa osatyössä käytimme suurta, kymmenen eri rotua sisältävää, aineistoa ja suoritimme analyysit sekä rotujen välillä että niiden sisällä. Vahvistimme yhteensä 21 perimän aluetta neljässätoista eri kromosomissa. Yksi näistä alueista ilmeni vain analyysissä, joka tehtiin yli rotujen. Vahvistetuilta perimän alueilta kootuista 254 kandidaattigeenistä tehtiin vuorovaikutusanalyysi, jossa havaitiin, että neddylaatio-polku oli rikastunut tässä geenisarjassa.

Tämä väitöskirjatutkimus on tuottanut merkittävää uutta tietoa lonkkaniveldysplasian sekä nivelrikon perinnöllisestä taustasta. Tuloksemme korostavat, että lievä lonkkaniveldysplasia eroaa tämän sairauden vakavammista muodoista perimän tasolla. Lonkkaniveldysplasiaan liittyvien perimän alueiden validointi on ollut harvinaista ennen tätä tutkimusta, joten useiden perimän alueiden vahvistaminen tässä väitöskirjatutkimuksessa oli huomattava edistysaskel tutkimusalalle. Myös kausaalisten perimän muutosten löytäminen on ollut hidasta. NOG-geenin häviämä-muutosten löytäminen ja niiden funktionaalisen merkityksen osoittaminen oli erityisen arvokasta, vaikka niiden kausaalisuus lonkkaniveldysplasiaan on vielä osoittamatta. Kaikkiaan tässä väitöskirjatutkimuksessa tuotettu tieto korostaa sitä, kuinka monimutkainen lonkkaniveldysplasian perinnöllinen tausta oikeasti on.

# Contents

# List of original publications

This thesis is based on the following publications:

I Mikkola LI, Holopainen S, Lappalainen AK, Pessa-Morikawa T, Augustine TJP, Arumilli M, Hytönen MK, Hakosalo O, Lohi H & Iivanainen A. Novel protective and risk loci in hip dysplasia in German Shepherds. *PLoS Genetics*. 2019 Jul;15(7):e1008197.

II Mikkola L, Holopainen S, Pessa-Morikawa T, Lappalainen AK, Hytönen MK, Lohi H & Iivanainen A. Genetic dissection of canine hip dysplasia phenotypes and osteoarthritis reveals three novel loci. *BMC Genomics*. 2019 Dec 27;20(1):1027.

III Mikkola L, Kyöstilä K, Donner J, Lappalainen AK, Hytönen M, Lohi H & Iivanainen A. An across-breed validation study of 46 genetic markers in canine hip dysplasia. *BMC Genomics*. In peer-review.

The publications are referred to in the text by their Roman numerals.

# Author's contribution

The author contributed to each publication as follows:

**I** **Novel protective and risk loci in hip dysplasia in German Shepherds**

*The author conceptualised and performed the experiments and data analyses, except the variant analyses and the dual luciferase assay (the author participated in designing and helped in executing the latter). The author drafted the manuscript with help from the supervisors and co-authors.*

**II** **Genetic dissection of canine hip dysplasia phenotypes and osteoarthritis reveals three novel loci**

*Apart from the radiographic phenotyping, the author conceptualised and performed the experiments and data analyses and drafted the manuscript with help from the supervisors and co-authors.*

**III** **An across-breed validation study of 46 genetic markers in canine hip dysplasia**

*The author conceptualised and performed the data analyses and drafted the manuscript with help from the supervisors and co-authors.*

# Abbreviations

| | |
|---|---|
| ADAMTS | A disintegrin and metalloproteinase with thrombospondin motifs |
| BMD | Bernese Mountain Dog |
| BW | Body weight |
| CFA | Chromosome for *Canis familiaris* |
| Chr | Human chromosome |
| CMH | Cochran-Mantel-Haenszel test for 2x2xK stratified tables |
| DAE | Dorsal acetabular edge |
| DI | Distraction index |
| DLS | Dorsolateral subluxation score |
| EBV | Estimated breeding value |
| EDTA | Ethylenediaminetetraacetic acid |
| FASTA | Family-based score test for association |
| FCI | Fédération Cynologique Internationale |
| FH | Finnish Hound |
| FHCDAE | Femoral head centre to dorsal acetabular edge |
| FIMM | Institute of Molecular Medicine Finland |
| FKC | The Finnish Kennel Club |
| FL | Finnish Lapphund |
| FRZB | Frizzled related protein |
| GD | Great Dane |
| GR | Golden Retriever |
| GS | German Shepherd |
| GWAS | Genome-wide association study |
| $h^2$ | Heritability (narrow sense) |
| HEVD | Hip-extended radiographs in ventrodorsal projection |
| HHIP | Hedgehog interacting protein |
| HWE | Hardy-Weinberg equilibrium |
| IGF-1 | Insulin like growth factor 1 |
| IL-1β | Interleukin 1 beta |
| KASP | Kompetitive allele specific PCR |
| KBD | Karelian Bear Dog |
| LAG | Lagotto Romagnolo |
| LD | Linkage disequilibrium |
| LI | Laxity index |
| LR | Labrador Retriever |
| MAF | Minor allele frequency |
| $M^2$ | Mantel-Haenszel statistics |
| MMP | Matrix metalloproteinase |
| Noa | Norberg angle |
| NOG | Noggin |
| OA | Osteoarthritis (when referred to the phenotype in Study II) |
| OFA | Orthopedic Foundation for Animals |
| OR | Odds ratio |
| PCR | Polymerase chain reaction |
| $r^2$ | Square of the correlation coefficient |
| ROS | Reactive oxygen species |
| QC | Quality control |
| QTL | Quantitative trait locus |
| QTSCORE | Fast score test for association |
| SAM | Samoyed |

| | |
|---|---|
| SNP | Single nucleotide polymorphism |
| STRING | Search tool for the retrieval of interacting genes/proteins |
| SWD | Spanish Water Dog |
| TGFβ | Transforming growth factor beta |
| TNFα | Tumour necrosis factor alpha |
| VMBDD | Vezzoni-modified Badertscher distension device |
| WGS | Whole genome sequencing |

# 1 Introduction

Studies of complex genetic disorders are becoming increasingly common. Different biobanks include large patient cohorts with good phenotype data. This is the case in both human medicine and veterinary sciences. A complex genetic background means that, in addition to the environment, numerous genetic variants impact the development of the disorder and most of the variants only have a small effect. To catch such variants, large sample sizes are needed. These are provided by the biobanks. In Finland, we have a canine DNA bank which holds over 70,000 samples. The dogs can have several phenotypes, from eye disorders to different skeletal diseases such as elbow and hip dysplasia.

Canine hip dysplasia is a major health issue that impacts the life of tens of thousands canine patients every year. The disorder develops in young puppies and commonly leads to the development of osteoarthritis, which is a painful and debilitating disease. Dogs affected by hip dysplasia and osteoarthritis may have reduced or, in the most severe cases, impaired movement of the hind limbs that will affect their daily lives. In addition to the negative implications hip dysplasia and possible osteoarthritis have on the welfare of the dogs, the economic burden on the owners and breeders is substantial.

Veterinary communities around the world are committed to controlling the disorder but they have limited means to do so as the disease aetiology is poorly understood. Although canine hip dysplasia is a complex disorder that is evidently affected by numerous genetic variants, uncovering the causal variants and related pathways can improve disease treatment options in the future. Hip dysplasia is also common in humans, and dogs have been proposed as a natural animal model for hip dysplasia in humans (1). Therefore, elucidation of the genetic factors of canine hip dysplasia and osteoarthritis may facilitate the advancement of human medicine.

Many years of research conducted by different research groups has highlighted that inadequate sample size is often the major limiting factor for discoveries in complex genetic disorders. Other major issues include the lack of validation of reported genetic associations and the heterogeneity of phenotypes within and between studies causing confounding and reducing statistical power. The current studies and results were facilitated by the canine DNA biobank and the phenotypes from the Finnish Kennel Club.

# 2 Review of the literature

## 2.1 Canine models of human inherited diseases

Humans and dogs (*Canis lupus familiaris*), descendants of the grey wolves (*Canis lupus lupus*), have a long, shared history. Dogs are considered to be the first domesticated species, but in spite of numerous studies investigating their evolution and domestication, when, where and how these processes took place has been disputed for many years (2,3). Nonetheless, Wang et al. (2016) (3) presented evidence demonstrating that our beloved companions originated from southern East Asia circa 33,000 years ago. Nowadays, there are over 400 distinct breeds with varying morphological characteristics and behaviour, which are a direct result of the artificial selective breeding humans have imposed upon dogs. There is evidence of some more general ancient dog classes (for example, herding dogs, sight hounds, and molossus) existing as long as 8000 years ago (2,4), but the strict breeding practices, and subsequently, most modern dog breeds are much younger – less than 200 – 300 years old (2,5,6).

The dog breeds are isolated breeding populations, meaning that usually there is no genetic mixing between breeds (6,7). Hence, the dogs within a breed have become phenotypically uniform and genetic diversity is restricted (6,7). The closed populations, population bottlenecks (such as those caused by World War II), and common use of popular sires has led to enrichment of disease-causing alleles and therefore the incidence of some hereditary disorders has increased (5–7). Many of these disorders have an analogue in humans (5–7), and both common and rare disorders are represented (5). To date the Online Mendelian Inheritance in Animals database has records for 439 potential canine models of human diseases, and over 260 mendelian traits/disorders with likely causal known variants in dogs (8). Humans and dogs not only share simple mendelian disorders, but there are myriad of analogous complex disorders, of which hip dysplasia is one of the most common.

Canine models of human disorders have been suggested to benefit from the higher physiological and clinical resemblance between dogs and humans in comparison to, for example, mice (5,9). In addition, the shared environment of dogs and their human owners is argued as another advantage in these canine models (5,7,9). What makes dogs especially useful for studies of genetic disorders is their unique genetic architecture. The canine genome embodies substantial linkage disequilibrium (LD); both ancestral short and recent long LD are present (5,10,11). Sutter et al. (2004) found that LD is up to a hundred times more extensive in dogs compared to humans (10). Therefore, the number of markers needed for genetic association studies can be substantially reduced in dogs (7,10,11), although long haplotype blocks may impede the recovery of the causal variants. Another approach is to use a high-density mapping array (12). Breed-specific variation in disease susceptibility and in the magnitude of within-breed LD is evident, but even the phylogenetically divergent breeds exhibit haplotype sharing (10). Hytönen & Lohi (2016) (5) stated that both within- and across-breed studies can facilitate novel gene discoveries. Although within-breed studies provide a powerful tool for disease mapping, Hayward et al. (2016) (12) also demonstrated the power of the across-breed approach. They found unprecedented loci for

complex disorders and morphological traits in a cohort of over 4200 individuals from more than 150 breeds and mixed-breed dogs using a high-density marker array (12). The steadily reducing prices of whole genome sequencing (WGS), although still quite high if deep coverage is needed, are bringing forth large WGS data sets such as the dog 10K project (13). These may increase the power of genetic studies in dogs, especially in the domain of complex genetic traits and disorders. However, the value of these data will be at least partially measured by the quality of the phenotypes linked with the data; without robust phenotypes, the utility of any genetic data is hindered. Furthermore, imputation of genomic sequence data (14) is becoming both more efficient and more popular. This may be the answer for smaller studies with fewer resources and without a consortium behind them while the price of WGS is still relatively high.

## 2.2 Target breeds in the study

### 2.2.1 German Shepherd

The history of the German Shepherd (**Fig. 1**) breed dates to 20 September 1899, when the breed standard was accepted in the first meeting of the "Verein für Deutsche Schäferhunde" (15). The German Shepherd population is genetically diverged into two main subpopulations (15,16), 'working line' and 'show line' (15). The split emerged during the 1960s and 1970s in Western Germany, when breeders started to put selection weight on different characteristics and purpose of use (15,16). Since the 1980s there has only been a limited change of genetic material between the main subpopulations (15). This is an important fact for genetic studies utilising German Shepherd populations because the population substructure will cause stratification issues (17), as was also observed and corrected for in the study of canine atopic dermatitis by Tengvall et al. (2013) (16).

**Figure 1.** ***German Shepherd.***
*Owner: Lea Mikkola.*
*Photo: Timo Jyllilä*

Hip dysplasia is a common veterinary problem in German Shepherds, which is the first breed that hip dysplasia was described in, by Schnelle in papers published in 1935 and 1937 (18–20). The Finnish Kennel Club (FKC) reports a prevalence of 37% (5952/16024) for hip dysplasia in German Shepherds among the screened dogs during the period 2000–2017 (year of birth) (21). When categorised by disease severity, the prevalence was 23% (3741/16024) for mild hip dysplasia, 11% (1805/16024) for moderate hip dysplasia, and 3% (406/16024) for the severe disease phenotype (21). In a recent Swiss study, the overall prevalence of hip dysplasia (including all disease categories) in a cohort of 5326 German Shepherds that were screened between 1995 and 2016 was ~32% (22).

However, the study reported a notable decrease in the disease prevalence between 1995 and 1999 and between 2010 and 2016, although the somewhat low rate of radiographing (31%) must be acknowledged in the interpretation of these results (22). The Orthopedic Foundation for Animals (OFA) reports breed-specific statistics of hip dysplasia from dogs that have been screened under the OFA system (in the United States). The statistics for German Shepherds, including evaluations until December 2018, reports that ~21% of 125,422 evaluated dogs were affected (23). This value however, as well as the reported prevalence from the FKC database, may be an underestimation of the real disease prevalence because neither the OFA nor the FKC screening system have a mandatory radiograph submission policy. It is suspected that many radiographs with obvious evidence of hip dysplasia are not sent for screening, which causes bias in the prevalence estimates (24–26). This bias concerns all breeds, not just German Shepherds.

The German Shepherd breed is one of the most popular globally and, because of the high disease prevalence and the abundance of dogs, German Shepherds have long been one of the main breeds of interest in studies on the genetics of canine hip dysplasia.

### 2.2.2 Other target breeds

Other target breeds in the study were the Finnish Lapphund, Golden Retriever, Lagotto Romagnolo, Bernese Mountain Dog, Samoyed, Spanish Water Dog, Great Dane, Labrador Retriever, Karelian Bear Dog, and Finnish Hound. Although these breeds represent very different types of dogs, all of them have a relatively high overall prevalence of hip dysplasia (21–45%), as reported by the FKC (**Table 1**). However, the examination rate within the breeds varies from as low as 10% in the Finnish Hound to 58% in the Samoyed (**Table 1**) (27–36). The breeds were selected for the study because of their high disorder prevalence and at least a cohort of 50 cases and 50 controls were available for each breed in the canine DNA bank at the University of Helsinki. Like the German Shepherd, the Labrador Retriever has been of special interest in studies investigating the genetic background of hip dysplasia, but Golden Retrievers and Bernese Mountain Dogs have previously been included in cohorts in such studies. To the author's knowledge, there has not been previous genetic studies on hip dysplasia for the rest of the breeds listed above.

**Table 1.** ***Breed-wise hip dysplasia statistics for ten different breeds in the period 2000–2017 (birth year)*** (27–36)**.**

| Breed | Percentage of screened dogs of the total dogs born | Overall prevalence of hip dysplasia | Prevalence of mild hip dysplasia | Prevalence of moderate hip dysplasia | Prevalence of severe hip dysplasia |
|---|---|---|---|---|---|
| FL | **34%** (6832/20187) | **35%** (2359/6832) | **27%** (1813/6832) | **8%** (517/6832) | **0.004%** (29/6832) |
| GR | **46%** (10785/23260) | **37%** (4006/10785) | **24%** (2606/10785) | **12%** (1265/10785) | **1%** (135/10785) |
| LAG | **51%** (2295/4489) | **45%** (1043/2295) | **31%** (707/2295) | **13%** (300/2295) | **2%** (36/2295) |
| BMD | **52%** (4290/8277) | **43%** (1849/4290) | **26%** (1125/4290) | **15%** (657/4290) | **2%** (67/4290) |
| SAM | **58%** (2297/3981) | **35%** (811/2297) | **22%** (515/2297) | **11%** (257/2297) | **2%** (39/2297) |
| SWD | **56%** (3192/5749) | **35%** (1112/3192) | **22%** (688/3192) | **12%** (384/3192) | **1%** (40/3192) |
| GD | **33%** (1736/5199) | **31%** (556/1736) | **25%** (439/1736) | **6%** (112/1736) | **0.003%** (5/1736) |
| LR | **53%** (15606/29357) | **21%** (3343/15606) | **13%** (2065/15606) | **7%** (1151/15606) | **1%** (127/15606) |
| KBD | **26%** (3576/13910) | **40%** (1458/3576) | **32%** (1145/3576) | **8%** (303/3576) | **0.003%** (10/3576) |
| FH | **10%** (3732/35678) | **29%** (1077/3732) | **20%** (745/3732) | **8%** (288/3732) | **1%** (44/3732) |

This data was acquired from the open database of the FKC on 29 November 2019. FL= Finnish Lapphund, GR= Golden Retriever, LAG= Lagotto Romagnolo, BMD= Bernese Mountain Dog, SAM= Samoyed, SWD= Spanish Water Dog, GD= Great Dane, LR= Labrador Retriever, KBD= Karelian Bear Dog, FH= Finnish Hound.

## 2.3 Hip dysplasia phenotypes in dogs

Hip dysplasia is defined as a complex, hereditary, and non-congenital disorder caused by abnormal joint development that usually leads to subluxation of the joint and subsequently to development of osteoarthritis (24,37). Increased hip joint laxity and delayed endochondral ossification are known to impact the development of hip dysplasia in dogs (24,37) but there is more to the disorder than what these factors can explain. This complexity of hip dysplasia, and especially the use of multifaceted phenotypes, has hindered genetic studies of the disorder. Hip joints, and therefore hip dysplasia and osteoarthritis, are comprised of a large number of traits that are not always easy to measure. Currently, hip dysplasia and osteoarthritis are often simplified into different categorical aggregate traits.

In Finland, the FKC has defined the guidelines for scoring hip dysplasia, as originally determined by the Fédération Cynologique Internationale (FCI) (38). The hip joints of a dog are categorically scored from A to E from hip-extended radiographs in ventrodorsal projection (HEVD) (39,40). Score A represents a normal hip joint and then the scores alphabetically progress to E, which corresponds to the most severely dysplastic hip joint.

The FCI/FKC score is a typical aggregate trait that consists of many sub-traits (**Table 2**). Within these hip scores, the most important aspect in evaluation for hip dysplasia is incongruity (a subjective trait), but the FKC veterinarian doing the scoring may also measure and evaluate other features of the joint. The Norberg angle, subluxation of the joint, shape and depth of the acetabulum, and how deep the femoral head centre sits in the acetabulum are some of the most commonly used indicators of hip dysplasia. Osteoarthritic changes are also assessed from the radiographs. Measuring these traits is subject to non-uniformity as veterinarians may evaluate the radiographic findings in different ways or emphasize their importance differently in the total score. Furthermore, the HEVD method is insensitive in diagnosing hip joint laxity (41–47). It must also be noted that the FCI screening system is not uniform throughout the FCI member countries. For instance, in Finland, both left and right hip scores are reported to the owner (e.g. A/A; left hip/right hip), while in many other countries only one score/grade, based on the worst hip, is reported.

**Table 2.** ***FCI scoring system used by the FKC in Finland***

| SCORE | DESCRIPTION |
|---|---|
| **A** | The femoral head and acetabulum are congruent. The craniolateral edge of the acetabulum is radiographically sharp and its form is mildly rounded. The joint cavity is uniform and tight. The Norberg angle in extension is approximately 105 degrees (recommended). |
| **B** | The femoral head and acetabulum are slightly incongruent and the Norberg angle in extension is close to 105 degrees, or the femoral head centre is positioned medially in relation to the dorsal acetabular edge and the femoral head and acetabulum are congruent. |
| **C** | The femoral head and acetabulum are incongruent, the Norberg angle is approximately 100 degrees and/or the craniolateral edge of the acetabulum is slightly shallowed. The cranial, caudal or dorsal edges of the acetabulum or the head or neck of the femur are uneven, or at most, some minor osteoarthritic changes are observed on them. |
| **D** | Notable unevenness on the femoral head and acetabulum. The joint is subluxated. The Norberg angle is more than 90 degrees (recommended). The craniolateral edge of the acetabulum is flattened and/or osteoarthritic changes are observed. |
| **E** | Clearly dysplastic hip joint. For instance, luxation or clear subluxation, the Norberg angle is less than 90 degrees, the craniolateral edge of the acetabulum is markedly flattened, the femoral head is deformed (mushroom-like, flattened) or other osteoarthritic changes are observed. |

There are also other screening systems for hip dysplasia. The following section describes some of the most common systems in addition to the FCI hip scoring scheme. The OFA uses the HEVD method to calculate an OFA hip score for dogs. This scoring system has seven categories. ranging from 'Excellent' to 'Severe'. The system is formed from nine sub-traits of the joint: craniolateral acetabular rim, cranial acetabular margin, femoral head morphology, fovea capitis, acetabular notch, caudal acetabular rim, dorsal acetabular margin, junction of femoral head and neck, and trochanteric fossa (48). Both the United Kingdom and Australia use the British Veterinary Association's (BVA) hip score, which also utilises the HEVD method. Numerical values are given to both hips and a total hip score, which is the sum of the separate hip scores (the larger the number, the more severe

the hip dysplasia) (49). The BVA score is an aggregate trait of nine sub-traits: Norberg angle, subluxation, cranial acetabular edge, dorsal acetabular edge, cranial effective acetabular rim, acetabular fossa, caudal acetabular edge, femoral head and neck exostoses, and femoral head recontouring (49).

The distraction index (DI) is a well-established and researched method for measuring hip joint laxity (PennHIP®) (42,43,50–52). Hip joint laxity is used to estimate the risk of developing osteoarthritis (also called degenerative joint disease, DJD) due to hip dysplasia. The PennHIP® method is estimated to be reliable due to the high between- and within-examiner repeatability (52). However, PennHIP® is a commercial system and an accredited veterinarian is required to radiograph the dogs. Although the method is popular in the US, accredited veterinarians are scarce in Finland and, in general, the method (or other laxity-based methods) has not yet become widespread in Europe (44,53). In addition, the costs associated with PennHIP® can be markedly higher compared to the traditional extension radiographs of hip joints (44). Nonetheless, the advantages of PennHIP® are undeniable. Comparison of the OFA hip score and the DI reveal that the OFA score does not fully entail the hip joint laxity, when some markable passive joint laxity was observed with the DI from dogs that were phenotyped as normal with the OFA score (43). Furthermore, another study investigated the agreement between the PennHIP® DI and the New Zealand Veterinary Association's hip scoring system, which is based on the HEVD method (54). The total scores from the HEVD scoring system compared poorly with the DI (54). It was also highlighted that the HEVD method was not well-suited for comparing individual dogs because of the low sensitivity and presence of false negatives (54). Kapatkin et al. (2004) compared the DI with a hip-extended index, where the laxity index was calculated from radiographs taken using the HEVD method (47). The DIs were consistently greater than the hip-extended indices, although a correlation of 0.52 was observed between the measures (47).

The Norberg angle, another measure of the state of joint subluxation and incongruity, has been criticized for being inaccurate as it is affected by the depth of the acetabulum. It has also been demonstrated that it is not a good predictor of osteoarthritis (42,51), that the somewhat arbitrary cut-off limit of 105 degrees for dysplastic/non-dysplastic hip joints is imprecise (55), and that its reproducibility is poor when there is no systematic agreement on the evaluation method between the observers (44). The position of the femoral head centre in relation to the dorsal acetabular edge (FHCDAE) is similarly used as an incongruity measure and it is highly correlated with the Norberg angle (56). In addition, the dorsolateral subluxation score (DLS) measures the femoral head coverage by the DAE (given as a percentage) in a natural weight-bearing position (57). This method has been demonstrated to be repeatable (58) and used quite widely.

A novel method for assessing hip joint laxity, named the Vezzoni-modified Badertscher distension device (VMBDD) technique (laxity index; LI), was described by Broeckx et al. (2018) (44). Comparison of the DI and LI, and the Norberg angle demonstrated that the VMBDD method is comparable to the PennHIP® in reliability, reproducibility (44), and in intra- and inter-observer variability (53). The technical repeatability and reproducibility of the VMBDD method was also demonstrated separately in a recent study (59). However, a study by Taroni et al. (2018) questioned how potential temporal variation of LIs may affect their interpretation and clinical significance (60). Although DI has previously been shown

to be stable over time (61), Taroni et al. (2018) used the VMBDD method, not PennHIP® (60). Even though DIs and LIs measured with PennHIP® and the VMBDD methods are highly correlated, there are differences in the overall procedures, which may play a role here, something Taroni et al. (2018) did not discuss. While this potential temporal variation should be investigated further, and comparison between the PennHIP® and VMBDD methods would be essential for this purpose, the VMBDD technique could prove a good solution for evaluating hip joint laxity reliably and affordably. This would lead to better estimations of osteoarthritis risk, and better efficiency for breeders selecting against hip dysplasia and osteoarthritis in comparison to the current FCI, OFA, and BVA scoring systems.

Finally, it must also be noted that the method of sedation/anaesthesia markedly affects the radiographic screening results of hip joints (62,63). Therefore, for example in Finland and Sweden, it is mandatory to record the type of the chemical restraint used in hip screening (39,62).

## 2.4 The hip joint

The abovementioned hip dysplasia phenotypes only approximate some parts of the hip joint anatomy and function. To understand the real complexity of the hip joint, it must be inspected more closely. Below the anatomy, development and normal biomechanics of hip joints, and ultimately the structure of articular cartilage, are described.

### 2.4.1 Gross anatomy and development

The coxofemoral (i.e. the hip joint) is a ball-and-socket joint (**Fig. 2**) where the head of the femur lies within the acetabulum and articular cartilage covers both of them, allowing smooth movement between the two structures (64,65). The femoral head attaches to the acetabulum with a strong ligament (*ligamentum capitis ossis femoris*) (64,66,67). This ligament proceeds from the fovea capitis of the femur to the acetabular fossa; the ligament is fully intracapsular (67) and a synovial membrane covers it (66). This ligament may also partly blend with another acetabular ligament (*ligamentum transversum acetabuli*) (67), which extends as the acetabular lip, while adhering to the bony acetabular edge and deepening the acetabulum with a fibrocartilaginous border (66). An articular joint capsule enfolds the femoral head and attaches to the femoral neck at one end and to the acetabular lip at the other (66). The joint capsule has two layers: a fibrous external layer and a synovial layer, which is infiltrated with blood vessels and nerves (64). The synovial membrane is responsible for producing the synovial fluid (synovia) lubricating the joint, thus reducing the friction between the articular surfaces of the femoral head and the acetabulum (64).

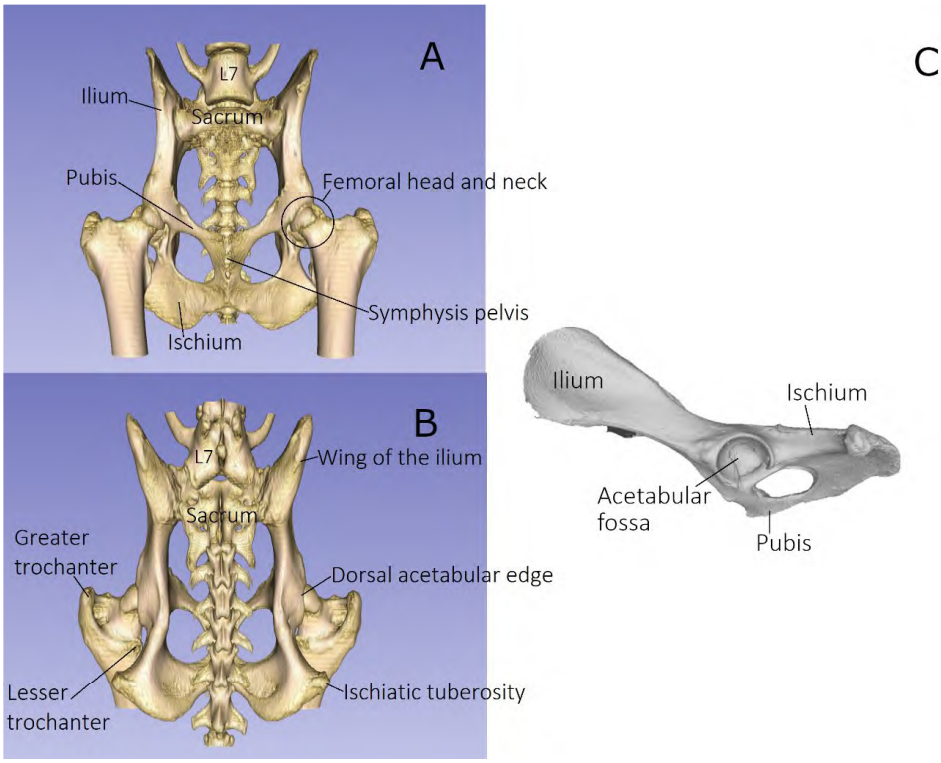


**Figure 2.** ***The pelvis and the hip joint of an adult German Shepherd****. **A)** The canine pelvis forms from two hip bones united by the symphysis pelvis midventrally. Dorsally, these bones articulate to the sacrum. The femoral head sits inside the acetabulum. **B)** There are multiple regions of attachment for muscles and ligaments in the pelvis and femurs, of which greater and lesser trochanter, ischiatic tuberosity, and parts of the wings of the ilia are some of the most prominent. The dorsal acetabular edge (DAE) holds the femoral head inside the acetabular socket. If the DAE is deteriorated, the hip joint may become unstable and the risk of subluxation increases. **C)** Each hip bone (os coxae) is a fusion of three primary bones: ilium, ischium and pubis, which also form the acetabulum. The acetabular bone is a triangular bone that incorporates the ilium, ischium and pubis during the growth period of the dog. The acetabular fossa is a fusion of the ischium and the acetabular bone; it is a non-articular surface and the femoral head is attached to it with a ligament. Image sources: A ventrodorsal view of a 3D reconstruction of a canine pelvis and hip joints from a CT scan (Lea Mikkola); A dorsoventral view of a 3D reconstruction of a canine pelvis and hip joints from a CT scan (Lea Mikkola); A lateral view of a 3D reconstruction of a canine pelvis (Sofia Kämäräinen).*

Normal development of a synovial joint happens through two main processes. First, a non-cartilaginous region called the interzone is formed for each future joint location (68,69). The interzone then becomes a crucial signalling centre for the opposing elements, which among other things regulate growth and express bone morphogenetic proteins and their antagonists (e.g. noggin) that are essential for normal joint formation (68). The second main process is cavitation that will lead to the physical separation of the skeletal anlagen of the joint and to the formation of the synovial cavity (68,69). Subsequently, the morphogenetic processes and differentiation of the cells lead to maturation of the joint, where the final interlocking shape and all the abovementioned structures are formed (69).

### 2.4.2 Normal biomechanics of the hip joint

Even though a ball-and-socket hip joint can move in any direction, the main movements of this joint are flexion and extension due to the opposing action of the medial and lateral rotator muscles (66). Flexion of the hip joint means reducing the angle between the femur and the acetabulum, whereas extension increases the angle and opens the alignment of the femur and the acetabulum (70). The other movements the hip joint is capable of are: adduction, abduction, circumduction, and outward and inward rotation (to some extent) (70). An unobstructed and smooth movement is essential for the normal function of the hip joint. If the conformation, and therefore stability, of the hip joint is compromised, abnormal biomechanical forces start to wear the structures of the acetabulum and the femoral head and neck.

Christen et al. (2014) studied the biomechanics of the canine, human and ancient feline hip joints. They estimated peak forces of 214% body weight (BW) on the canine hip joint, and 458% BW for a human hip joint (71). The loading patterns of human and canine hip joints differed in that the main forces concentrated on the superior end of the femoral head in humans, but in dogs the forces started superiorly and then spread medially (71). Thus, as expected, the quadrupedal vs. bipedal movement affected the loading: the main forces were more uniformly spread over the femoral head in dogs (and ancient felines) than in humans (71).

The importance of the biomechanics of the hip joint, in addition to the possible wear and tear due to compromised joint stability, is that bone development and remodelling is affected by mechanical stimuli. Bone tissue is dynamic, meaning continuous renewal, which is affected by hormones, cytokines and the mechanical environment of the bone (72). 'Wolff's law' on bone transformation from 1892 (73) has been the gold standard for how bone responds to mechanical stimuli during osteogenesis and remodelling.

> Every change in the form and function of a bone, or of function alone, is followed by certain definite changes in the internal architecture and equally definite secondary alterations in the external conformation in accordance with mathematical laws.
>
> *Wolff 1892*

However, Wolff's law has also been disputed (74,75) and criticized of being too simplistic (75,76). One argument is that there are important differences in how loading impacts bone

at different stages; that is, bone differentiation and morphogenesis, bone growth, and ultimately, bone homeostasis in adult individuals (77).

Mechanical stimuli play a role already during the initiation of the skeletal growth (78). For example, the mesenchymal progenitor cells turn to adipocytes in the absence of mechanical stress, but differentiate to chondroblasts in response to changes in compression (78). It is also known that the synthesis of hyaluronic acid (hyaluronan), an important component of articular cartilage, responds sensitively to mechanical stress in chondrocytes (79,80). Subsequently, during the prenatal growth of bones, the skeleton of a foetus is modified by the mechanical environment of the uterus (81). It is important that the mechanical stress induced on the bone is within moderation as high intensity pressure is known to inhibit bone growth (77). However, adult bone is normally able to adapt to the alteration in the biomechanical loading to some extent. This results from the coupled remodelling processes of osteoblasts and osteoclasts, where new bone is formed or old bone resorbed (72,82), striving to maintain a uniform load on the bone surfaces (71), and repairing possibly damaged bone tissue (72).

### 2.4.3 Articular cartilage

As previously explained, the epiphyseal subchondral bone in the bones of the hip joint is covered by articular (hyaline) cartilage (64,65). Articular cartilage has no nerves and it is also avascular (65,70). It is composed of an extracellular matrix (ECM) which is sparsely infiltrated with chondrocytes, accounting for approximately 2% of the total volume (65). Ultimately, articular cartilage is quite a complex structure, organised in distinct zones (**Fig. 3**) (83).

The superficial tangential zone is formed from elongated chondrocytes that are oriented parallel to the articular surface and produce the anti-adhesive lubricin, which creates the frictionless movement of the joint (83). The middle (i.e. the transitional zone) contains more roundly shaped chondrocytes organised in vertical rows, but more randomly than in the superficial zone. They produce and thus maintain the components of the ECM (83). The deep zone is organised perpendicular towards the surface and consists of larger chondrocytes that actively participate in the production and maintenance of the ECM components (83). The chondrocytes in the deep zone face the tissue boundary called the tidemark (**Fig. 3**) (83), which separates the articular cartilage from the calcified zone (84). The calcified zone, where chondrocytes are in a hypertrophic state in calcified ECM, is connected to the subchondral bone (84). Interestingly, the superficial zone has been shown to contain stem/progenitor cells, which have been hypothesised to participate in cartilage repair (84,85). These chondroprogenitor cells have also been found within the other zones (and other tissues adjacent to the articular cartilage surface), but in much smaller quantities (85).

Two of the major components of articular cartilage are hyaluronan and aggrecan. Aggrecan units comprise an aggrecan core protein and chondroitin sulphate and keratan sulphate side chains. Aggrecan molecules and link proteins form a complex with a single hyaluronan molecule to form a large cartilage proteoglycan aggregate (86). These proteoglycan aggregates maintain the highly hydrated state of the ECM (87). Water is one

of the major components of ECM (68–85% of the total weight) (65,88). Other components of the articular cartilage are collagens (about 60–86% of the dry weight), of which type II collagen is the most ubiquitous, proteoglycans (from 15–40% of the dry weight) like the abovementioned aggrecan, but also biglycan, decorin and fibromodulin, in small quantities, and then other non-collagenous proteins (e.g. cartilage oligomeric matrix protein) (65,88) (**Fig. 3**). Of these, the proteoglycan aggregates, and the water that is drawn into cartilage by a negative charge, are the key factors in sustaining the mechanical properties of the cartilage, meaning its capability to resist compression (65,88,89).

Chondrocytes maintain the articular cartilage of synovial joints (69,90). They synthesise ECM components and matrix degrading enzymes, but with minimal turnover of the cells and ECM (90). The chondrocytes within articular cartilage are in an arrested pre-hypertrophic state and do not continue differentiation, thus enabling them to continue the processes that maintain normal articular cartilage structure (69,90).

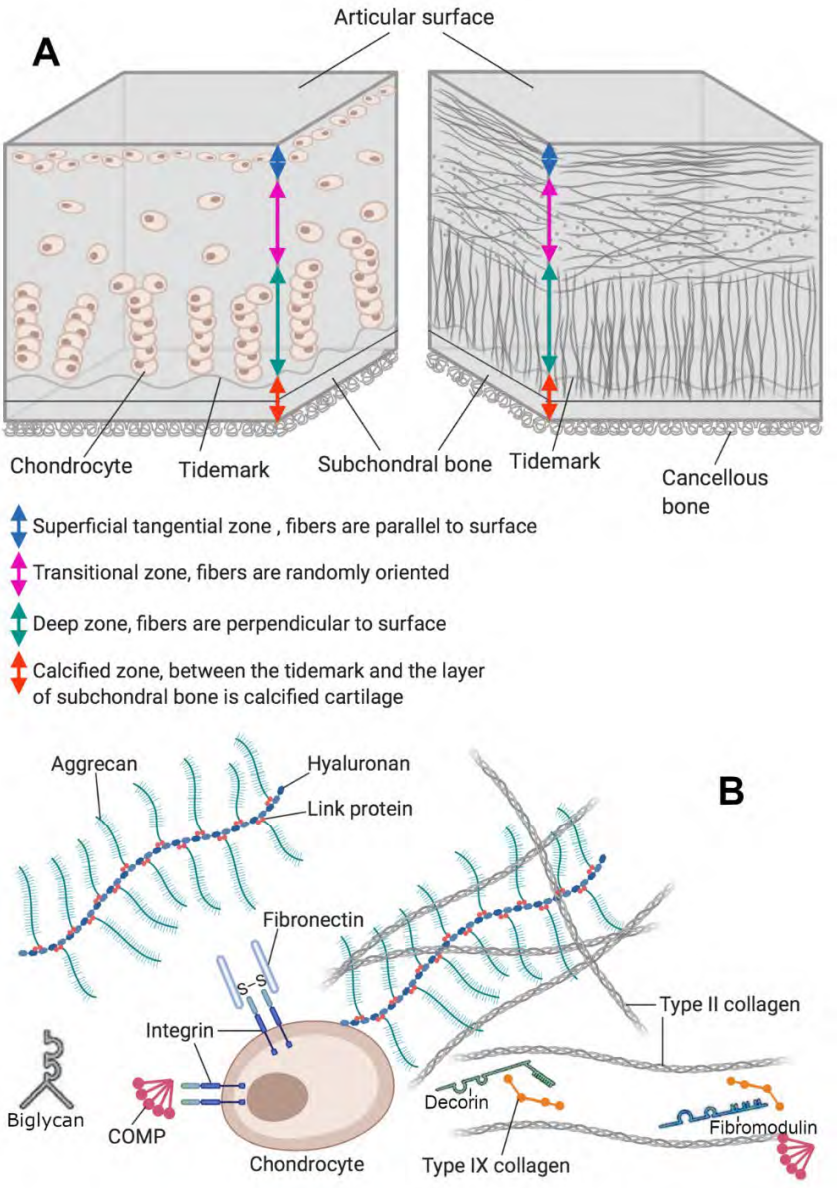


**Figure 3.** ***Articular cartilage structure and composition.*** *A) Schematic of a cross-section of articular cartilage with cellular organisation on the left and collagen fibre architecture on the right. B) Cartilage ECM with its components. COMP= cartilage oligomeric matrix protein. Created with BioRender; modified from Chen et al. (2006)* (88) *and Fox et al. (2009)* (65).

## 2.5 Hip dysplasia is a complex hereditary disorder

As described in the previous sections, a hip joint is a complex ensemble of different structures, tissues and cell types. A myriad of different genes and signalling pathways play roles in the normal genesis and homeostasis of these complexes. Thus, there are also many ways the development, function or maintenance of the hip joints can go wrong, and osteoarthritis can follow. The multifactorial and non-congenital background of canine hip dysplasia is well-established (24,37,91–93). Although there is still uncertainty how the disease arises in dogs, decades of research in this area have revealed some environmental and genetic factors that impact the development of hip dysplasia in dogs.

### 2.5.1 Environmental factors

Understanding how the environment may affect disease development is important, especially in genetic studies to limit the confounding that environmental effects may create in a complex disease. In addition, from the perspective of dog owners, understanding the environmental components can lead to better management, and thus lowered risk, of disease development in an individual if the environmental factors are controllable by the owner.

#### *2.5.1.1 Nutrition*

Environmental factors do not cause the disorder by themselves, but they have an impact on how hip dysplasia develops in genetically predisposed dogs. The role of nutrition in the development of hip dysplasia has been studied to some extent. Excess of certain nutrients rather than their deficiencies have been reported to influence the development of hip dysplasia (37,94) while variation in the protein or carbohydrate content of food does not affect development of hip dysplasia, as long as the protein and amino acid content is sufficient for growth (37,94).

Excessive calcium has been implicated as a nutritional risk factor for hip dysplasia in large growing dogs. The concentration of calcium in plasma is highly regulated in dogs because, in addition to its obvious role in the bone, there are many other calcium-dependent processes, such as muscle contraction and blood clotting (94) that would be disturbed without this control. In puppies, a high intake of dietary calcium leads to increased amounts absorbed because the mechanism that should protect the dog against excess calcium absorption is not yet active (24,37,95–97). As a result, osteoclast activity is hampered, which affects endochondral ossification and bone remodelling (37,94,97,98). Moreover, because vitamin D increases the absorption of calcium from the gastrointestinal tract and its renal resorption, excessive supplementation of vitamin D may lead to similar adverse effects (37,94).

The effects of dietary anion gap on the development of hip dysplasia has been investigated (37,94,99). It was found that the osmolality of the synovial fluid was higher in dysplastic dogs in comparison with non-dysplastic dogs (37,99). The higher osmolality results in different electrolyte concentrations of sodium, potassium and chloride in the

synovial fluid (37,99). It was suggested that the severity of hip joint laxity could be reduced by controlling the dietary anion gap or, in other words, dietary electrolyte balance in growing dogs (94,99). Other than the abovementioned factors, the impact of nutritional elements on the development of hip dysplasia are not well known.

#### *2.5.1.2 Exercise*

Studies on the relationship between exercise and hip dysplasia are scarce. Krontveit et al. (2012) investigated the housing- and exercise-related risk factors of hip dysplasia (100). The same study cohort was used as in (101). The results were limited to the first three months of life after birth, but two exercise conditions were significantly associated with the risk of hip dysplasia: daily use of stairs until three months of age (increased risk) and daily off-leash exercise in a park until three months of age (protective effect) (100). They concluded that puppies less than three months old should not have access to stairs, but that off-leash outdoor exercise in a park-like terrain could protect from hip dysplasia (100). The latter, they suggested, could be a result of better muscle development and increased strength in the hips (100).

Greene et al. (2013) found that exercise duration was significantly inversely associated with lameness scores in dysplastic dogs (102). This means that the dogs with longer daily exercise times were less lame than dogs that got less exercise. Interestingly, the type of exercise (i.e. high or low impact) was not associated with the lameness scores (102). This suggests that as long as a dysplastic dog gets enough exercise, it may benefit from it, and at the same time, dysplastic dogs seemed not to be adversely affected, even if they were participating in some heavy exercises such as jogging/running, hunting or even field trials.

#### *2.5.1.3 Biomechanical alterations during foetal development*

Breech position of the foetus (the foetus does not turn head down before birth), some neuromuscular disorders and oligohydramnios (deficiency of amniotic fluid, which limits the intrauterine space) are known to increase the risk of developmental dysplasia of the hip in humans because they restrict the normal movement of the hip joint (103,104). Nowlan et al. (2014) found that foetal immobilization in chick embryos induced flattening of the femoral head, abnormal pelvic orientation in relation to the femur, abnormal positioning of the acetabulum, and reduced acetabular coverage of the femoral head (104). Their findings demonstrate how abnormal prenatal movement can influence the development of the hip joint in its early stages. These finding are also consistent with the impact of pressure and mechanical stimuli on growing bone and articular cartilage components (77,78,81), as previously described in paragraph 2.4.2. Such developmental evidence is lacking in dogs. It is important to note that hip dysplasia in dogs is not apparent at birth. However, subtle changes in hip morphology by the intrauterine environment could still be possible, while the adverse structural and/or functional effects may manifest only after the hip joint is

influenced by weight-bearing. The effects of these factors on the development of canine hip dysplasia are yet to be identified.

#### *2.5.1.4 Weight-related risk factors*

Vanden Berg-Foels et al. (2006) demonstrated that high birth weight was associated with reduced coverage of the femoral head at four months of age and with increased probability of degenerative changes at eight months compared to puppies with lower birth weights (105). Higher birth weight was also negatively associated with the onset of femoral head ossification, but it was suggested that this was likely due to differences in skeletal maturity (105). However, it must be considered that birthweight itself is a result of prenatal growth and skeletal maturity, which are affected by genetic factors and intrauterine environmental effects (105).

Several studies have implicated rapid weight gain as a risk factor for developing hip dysplasia (37,94,106–109). However, not all research corroborates this, as was demonstrated by Lopez et al. (2006) (110) and Krontveit et al. (2010) (101). Lopez et al. (2006) investigated the relationship between juvenile weight gain and joint laxity, measured with PennHIP® at 16 weeks of age in puppies fed *ad libitum* and found no association (110). Krontveit et al. (2010) found that higher body weight at three months of age had a modest protective effect on hip dysplasia in the study cohort, which included dogs of three giant breeds and Labrador Retrievers (101). It is not fully clear what can explain these opposing findings, but Lopez et al. (2006) suggest the contradiction is due to dissimilarities in study designs, phenotypes and cohorts (110). Krontveit et al. (2010) suggest that one reason may be the different genetic risk in the study cohorts (101). While many of the dogs in earlier studies were the progeny of dysplastic parent(s) and thus probably entailed higher genetic risk, this was not the case in the study cohort in (101). Krontveit et al. (2010) also emphasise that different environments (controlled, standardised or private homes), different breeds, change in the dog foods used, or change in the conformation of the breeds over the past few decades, may explain some of the discrepancies between the studies, because many of the previous studies were conducted 20–30 years ago (101).

Three studies conducted with Labrador Retrievers revealed that limited food consumption in comparison to feeding the dogs *ad libitum* has significant effects on the development of hip dysplasia and osteoarthritis (109,111,112). Excessive food consumption was determined a risk factor, and reciprocally, limited food consumption resulted in substantially reduced prevalence of hip dysplasia and osteoarthritis (109,111,112). Furthermore, Smith et al. (2006) conducted a lifelong longitudinal study with the same dogs that were used in (109,111,112) on how diet restriction affects the development of radiographic evidence of hip joint osteoarthritis (113). They found that restricted feeding ‘had a profound effect on the radiographic hip phenotype’ (i.e. the development of osteoarthritis was markedly delayed in this group) (113). It was also highlighted that dogs susceptible to hip dysplasia should be kept lean throughout their lives (113).

Brady et al. (2013) demonstrated that obesity in dogs impacts the joint kinematics at the sagittal plane (114). They found that, when compared to their lean counterparts, the stance phase range of motion (ROM) at two different trotting velocities was significantly greater

in the hips of obese dogs (114). A similar effect was observed in elbows, shoulders and tarsal joints (114). The swing phase ROM was significantly greater in obese dogs, and increased velocity associated with increased stance and swing ROM in these dogs (114). The peak vertical ground reaction forces, as well as the horizontal ground reaction forces of fore- and hind limbs were significantly greater in obese dogs compared to lean dogs (114). Brady et al. (2013) suggest that these changes in the joint kinematics of obese dogs may increase the susceptibility of developing osteoarthritis (OA), especially if the joints have intrinsic laxity, because the consequent subluxation could be worsened by the increased ground reaction forces and ROM (114). These studies accentuate how obesity in dogs may affect the development of hip dysplasia and subsequent osteoarthritis, and why keeping dogs in lean body condition is imperative.

#### *2.5.1.5 Hormonal factors*

The possible contribution of hormonal factors to the development of hip dysplasia have been investigated over many years and the studies have led to different and partially inconsistent findings. Oestrogen, especially estradiol-17β, and relaxin are the most studied hormonal factors in canine hip dysplasia.

Pierce & Bridges (1967) studied the role of estradiol in the pathogenesis of hip dysplasia by injecting 'a flooding dose' of estradiol-17β intramuscularly to genetically predisposed puppies and their counterparts from non-dysplastic lineages (115). They observed differences in the urinary estradiol-17β quantities between the case and control groups, suggesting that dogs with hip dysplasia may have altered oestrogen metabolic pathways that result in reduced capacity to metabolise estradiol-17β, thus leading to its increased urinary excretion (115). However, it must be noted that the level of endogenous oestrogen in dysplastic puppies is not any different to non-dysplastic dogs (24,37), and Pierce & Bridges (1967) also stated that, prior to the exogenous estradiol-17β injections, the urinary estradiol concentrations were unmeasurable in both groups (115).

Around a decade later, Gustafsson (1975) (116), Kasström et al. (1975) (117) and Beling et al. (1975) (118) continued to investigate the effects of estradiol on hip dysplasia in different breeds. Gustafsson (1975) demonstrated that exogenous estradiol benzoate administered either to Beagle puppies postnatally or to pregnant bitches caused adverse changes such as decreased size of the femoral head and hip joint instability (116). Changes in the femoral head ossification centre and in the bone mass (increased or decreased) were observed in the case groups (116). Similarly, Steinetz et al. (2008) found that transmission of milk-borne maternal hormones (relaxin and oestrogen/oestrogen precursors) to suckling pups may have played a role in the development of hip dysplasia in genetically predisposed Labrador Retriever puppies (119). Decreased femoral head size was also observed in this study (119).

Kasström et al. (1975) measured peripheral plasma levels of estradiol in different breeds and examined the ability of canine plasma to bind dihydrotestosterone and estradiol (117). They found significant difference in the estradiol levels of dysplastic and non-dysplastic German Shepherds (117). However, they concluded that it is unlikely that hyperoestrogenism would be an etiological factor in hip dysplasia; instead, they suggested

that the plasma proteins that bind steroids could play a role in the development of hip dysplasia (117). Beling et al. (1975) studied the metabolism of oestrogen in dysplastic adult German Shepherds and non-dysplastic adult Greyhounds (118) and found that the majority of the administered exogenous estradiol-17β was unmetabolised and excreted in all dogs, and that the levels of the parts that were metabolised were similar regardless of the phenotype or gestation status of the dogs (118). Their conclusion was that the capacity of dysplastic dogs to metabolise and eliminate excess oestrogen or its metabolites was not impaired (118). Beling et al.'s (1975) (118) findings are discordant with the conclusions of Pierce & Bridges (1967) (115), although the research in (118) was conducted with adult dogs while (115) included only puppies, which may affect the interpretation. Indeed, Steinetz et al. (2008) suggest that the hip joint laxity they observed may have resulted from premature or otherwise inappropriate expression of oestrogen and/or relaxin receptors in the connective tissues of hip joints of the genetically predisposed puppies, while the measured total oestrogen/relaxin concentrations were similar in all the adult dams (119). Therefore, the role of oestrogen and especially estradiol-17β, in the development of hip dysplasia demands further research.

Both Goldsmith et al. (1994) (120) and Steinetz et al. (2008) (119) studied the role of relaxin in hip dysplasia, although Steinetz, Goldsmith & Lust had already suggested the potential role of relaxin in canine hip dysplasia in 1987 (121). Goldsmith et al. (1994) noted that, in addition to the relaxin that must have been transmitted to the puppies *in utero*, the hormone was transmitted from the dams to the suckling puppies via milk (120). Relaxin extracted from dogs caused pelvic relaxation in mice and therefore Goldsmith et al. (1994) suggested that relaxin may also take part in the development of hip joint laxity (120). Later, Steinetz et al. (2008) tested this hypothesis and concluded that milk-borne hormones may contribute to the development of hip joint laxity in predisposed puppies (119). However, the effects of endogenous and milk-derived oestrogen/estradiol and relaxin levels could not be interpreted separately in Steinetz et al.'s (2008) study.

Hashem et al. (2006) compared the effects of exogenous relaxin, estradiol-17β and progesterone, alone or in combinations, on the glycosaminoglycans and collagens of the temporomandibular joint disc and knee meniscus fibrocartilages, knee articular cartilage and the pubic symphysis in rabbits (122). Estradiol-17β and relaxin alone, and the combination of relaxin and estradiol-17β resulted in significant loss of glycosaminoglycans and collagen from the pubic symphysis, temporomandibular disc, and loss of collagen from the knee articular cartilage, but not from the knee meniscus (122). Administration of progesterone protected against these effects. Their findings suggest that the loss of ECM components by different hormones may selectively participate in the degeneration of certain synovial joints (122). This is yet to be proven in the articular cartilage of canine hip joints.

#### *2.5.1.6 Other environmental factors*

Several studies have investigated the effects of sex and, in some, the effects of neutering on the risk of developing hip dysplasia. The findings from these studies have been divergent (93). Many studies have reported no difference between the two sexes (93). For instance, Wood et al. (2000) (123), Krontveit et al. (2010) (101), and Freeman et al. (2013) (124)

found no significant sex effects in their cohorts of varied large and giant breeds, while Loder & Todhunter (2017) observed a slightly increased prevalence of hip dysplasia in females, yet the effects were breed-dependent (93). Hedhammar et al. (1979) reported a 14% increase in the prevalence of hip dysplasia in female German Shepherds over males (125).

In Golden Retrievers, sex differences were observed and what is noteworthy in this study is that early neutering of males had a significant effect on the development of hip dysplasia (126). This was suggested to result from the combined effects of neutering on the growth plates and increased weight (126). Spain et al. (2004) studied the long-term risks and benefits of neutering at an early age (younger than 5.5 months) (127). They reported increased risk of hip dysplasia in dogs that were neutered at an early age regardless of the sex (127). Interestingly, they also observed that despite the increased risk of hip dysplasia in these dogs, they were less likely to be euthanised due to the condition in comparison to the dogs that had been neutered at an older age and had developed hip dysplasia (127). They postulated, with some reservations, that the early neutering may be associated with a milder form of hip dysplasia (127).

Seasonal effects have been reported to affect the risk of developing hip dysplasia in dogs (93). The results vary between studies but, for example, in Krontveit et al.'s (2012) study in Norway, the odds ratio (OR) for hip dysplasia was two to three times higher (depending on the classification of the season) for dogs born in autumn compared with dogs born in the winter (100). The odds for dogs born in the summer being affected by hip dysplasia were about half compared to dogs born in the winter, while the ORs for puppies that were born in spring (compared to winter puppies) varied between 0.52 and 1.3, depending again on the classification of the season (100). The change in the spring effect was explained by the slow start of thermal spring in Norway (100). In a Finnish cohort of German Shepherds, the dogs that had been born during spring or summer had less hip dysplasia (128). Loder & Todhunter (2017), who, in addition to their own study, reviewed the seasonal effects from many earlier studies, concluded that dogs born in the autumn or winter months in the Northern Hemisphere had a higher prevalence of hip dysplasia (93). In their own analysis of 619,825 dogs, they observed seasonal variation, where prevalence of hip dysplasia was slightly increased in dogs born in the spring and winter months (93).

It is unclear what causes the seasonal variation in the prevalence of hip dysplasia, but many reasons have been suggested. One reason could be the muscular development of dogs, which may be affected by the season if puppies born during the late autumn or winter get less exercise (93). This could result in weaker muscles that offer inadequate support to the hip joints and lead to hip dysplasia (93,100,101,129,130). Loder & Todhunter (2017) (93) suggest diet and weight gain as other sources of the seasonal variation. More specifically, vitamin D deficiency during winter, as well as other dietary factors that may fluctuate seasonally, and disproportion between the food intake and level of activity during the winter months are suggested (93).

### 2.5.2 Genetics of canine hip dysplasia

The first studies investigating the heritable components of canine hip dysplasia did not have molecular genetic tools or data. They utilised phenotypes, environmental and pedigree

information of a given population to determine different variance components for genetic, environmental and phenotypic variation. Subsequently, heritability can be estimated from these variance components. Heritability is an important statistical concept in quantitative genetics. It estimates the degree to which genotypic differences (variation) in individuals explain the differences in observed phenotypes. The principles of heritability, as we know them, were originally introduced by Sewall Wright and Ronald A. Fisher in the 1920s (131). Narrow sense heritability ($h^2$) is calculated as the division of additive genetic variance by the phenotypic variance (132). A heritability estimate is population, environment and time specific (132). This means that a heritability estimate for one breed may be different given a different environment (e.g. country). Temporal incompatibility of population-wise $h^2$ estimates may arise if the first population estimate is old and many generations of selective breeding have led to changes in allele frequencies, or the environmental factors have changed considerably during that time. Moreover, estimation methods affect the heritability estimates.

There are numerous heritability estimates for canine hip dysplasia in different breeds and their populations, at different times and with different hip dysplasia phenotypes. One of the first studies that reported a heritability estimate ($h^2$=0.22) for a hip dysplasia score in a German Shepherd population was carried out by Leighton et al. (1977) (133). Hedhammar et al. (1979) studied a Swedish cohort of 401 German Shepherd litters from the Swedish Armed Forces (125). The heritability of hip dysplasia in this population was estimated to be between 0.40 and 0.50 (125). Leppänen et al. (2000), Mäki et al. (2000) and Mäki et al. (2002) estimated heritabilities for hip dysplasia (FCI hip score) for different breeds in Finland, which are especially relevant for this thesis. First, Leppänen et al. (2000) estimated the heritability of hip dysplasia in a Finnish German Shepherd cohort and found it to be moderate; $h^2$ was between 0.30 and 0.35 from models with different random environmental effects (134). Mäki et al. (2000) conducted a similar quantitative study in Finnish Rottweilers and reported a notably higher heritability estimate for hip dysplasia in this breed ($h^2$= 0.58) (135). Later, Mäki et al. (2002) investigated several breeds and reported heritability estimates from three models that included either all dogs within a breed, or only females or only males (136). The heritability estimates between sexes were different in Golden Retriever, Rough Collie and Bernese Mountain Dog, although none of the differences were statistically significant (136). The breed-wise heritability estimates that included both sexes varied between 0.20 (Rough Collie) and 0.41 (Finnish Hound). Interestingly, heritability estimates for both German Shepherds and Rottweilers were lower in this study than in the previous studies mentioned above (0.24 and 0.38, respectively) even though partially the same cohorts were used (136). This may have been due to the marked increase in the number of screened dogs between the studies (136).

At present, heritability estimates are still invaluable sources of information. They can be used to estimate breeding values or predict response to selection, of which the first (usually designated estimated breeding values; EBVs) are currently the most efficient way to select against hip dysplasia in breeding programs (137,138). Some recent heritability estimates are presented in **Table 3.**

**Table 3.** ***'Present-day' estimates of heritability of canine hip dysplasia phenotypes in different populations from few different studies.***

| Estimated heritability ($h^2$) (SE) | Hip dysplasia phenotype | Breed(s) | Estimation method | Reference |
|---|---|---|---|---|
| 0.59 (0.13)$^{1A}$, 0.27 (0.11)$^{2A}$, 0.29 (0.11)$^{3A}$, 0.52 (0.12)$^{4A}$, 0.44 (0.12)$^{5A}$, 0.28 (0.10)$^{6A}$, 0.23 (0.10)$^{7A}$, 0.36 (0.10)$^{8A}$, 0.19 (0.10)$^{9A}$, 0.06 (0.08)$^{10A}$, 0.15 (0.10)$^{11A}$; 0.23 (0.06)$^{1B}$, 0.25 (0.06)$^{2B}$, 0.15 (0.05)$^{3B}$, 0.36 (0.06)$^{4B}$, 0.27 (0.06)$^{5B}$, 0.21 (0.06)$^{6B}$, 0.18 (0.06)$^{7B}$, 0.28 (0.06)$^{8B}$, 0.20 (0.06)$^{9B}$, 0.11 (0.05)$^{10B}$, 0.18 (0.06)$^{11B}$ | 1= HS<br>2= THS<br>3= Noa, right<br>4= Noa, left<br>5= Noa, total<br>6= SUB, right<br>7= SUB, left<br>8= SUB, total<br>9= CrAE, right<br>10= CrAE, left<br>11= CrAE, total | LR (UK) | REML used on either<br>A=pedigree data or<br>B=genomic data | Sánchez-Molano et al. (2014) (139) |
| 0.81 (0.027)$^{1aM}$, 0.74 (0.038)$^{1bM}$, 0.42 (0.061)$^{1cM}$; 0.63 (0.037)$^{2aM}$, 0.76 (0.032)$^{2bM}$, 0.60 (0.056)$^{2cM}$; 0.76$^{1aB}$, 0.72$^{1bB}$, 0.41$^{1cB}$; 0.60$^{2aB}$, 0.66$^{2bB}$, 0.59$^{2cB}$ | 1= hip-extended score<br>2= DI | a= GS (US, purpose-bred)<br>b= LR(US, purpose-bred)<br>c= GR (US, purpose-bred) | Two methods<br>M= MTDFREML<br>B= Bayesian MCMC | Leighton et al. (2019) (140)ˣ |
| 0.58 (0.03)$^{a}$, 0.47 (0.02)$^{b}$, 0.55 (0.03)$^{c}$, 0.52 (0.03)$^{d}$, 0.59 (0.05)$^{e}$, 0.65 (0.03)$^{f}$ | OFA hip score | a= GS<br>b= BMD<br>c= GD<br>d= SAM<br>e= LR<br>f= GR | Bayesian MCMC | Oberbauer et al. (2017) (141)* |
| 0.24 (0.15)$^{a}$, 0.73 (0.21)$^{b}$, 0.28 (0.14)$^{c}$ | Average Norberg angle score | a= LR (UK)<br>b= LR (Cornell)<br>c= LR (combined data) | AI-REML | Edwards et al. (2018) (142)ǂ |
| 0.20 (SD=0.05; CI=0.12-0.31)$^{1}$, 0.31 (SD=0.05; CI=0.21-0.41)$^{2}$ | 1= FCI hip score<br>2= Hip status (normal or dysplastic) | GS (Brazil) | Gibbs sampling for threshold linear mixed models | Babá et al. (2019) (143) |
| 0.81 (0.40)$^{1}$, 0.35 (0.36)$^{2}$, 0.15 (0.28)$^{3}$, 0.53 (0.36)$^{4}$ | 1= Transformed DI left hip<br>2= Transformed DI right hip<br>3= Transformed DI worse hip<br>4= Transformed DI average | GS (New Zealand, purpose-bred) | ASReml | Tikekar et al. (2018) (144) |
| 0.28 (0.03)$^{1a}$, 0.28 (0.01)$^{2a}$, 0.41 (0.01)$^{3a}$,<br>0.15 (0.02)$^{1b}$, 0.29 (0.01)$^{2b}$, 0.34 (0.01)$^{3b}$ | 1= Transformed FCI score (France)<br>2= Transformed FCI (Sweden)<br>3= Transformed BVA score | a= GR<br>b= LR | AI-REML | Wang et al. (2017) (145) |

Apart from (139), the studies were conducted with pedigree-based data. ˣ Leighton et al. (2019) used two different methods to estimate the genetic parameters, and standard errors were only calculated with the REML method (140). The hip-extended score in their study is acquired as part of the

PennHIP® method. * Oberbauer et al. (2017) had heritability estimates from 60 different breeds in the US (141), so only the ones that have also been studied in this thesis are listed here. ǂ Edwards et al. (2018) used an average Norberg angle score as their phenotype, which is not the same as a basic Norberg angle, but is derived from it (142). They also reported heritabilities estimated as part of cross validations, reported as average heritabilities; only the Cornell cohort had a different estimate ($h^2$= 0.66) (142). SE= Standard error; for one study standard deviations with 95% confidence intervals were reported instead, which are marked with SD and CI respectively. HS= Hip score from the British Veterinary Association; BVA, THS= Transformed hip score, Noa= Norberg angle, SUB= Subluxation, CrAE= Cranial acetabular edge, DI= Distraction index. OFA= Orthopedic Foundation for Animals, FCI= Fédération Cynologique Internationale. LR= Labrador Retriever, GS= German Shepherd, GR= Golden Retriever, BMD= Bernese Mountain Dog, GD= Great Dane, SAM= Samoyed. REML= Restricted maximum likelihood, MTDFREML= Multiple trait derivative free REML, MCMC= Markov chain Monte Carlo method, AI-REML= The average-information REML, ASReml= Average information and sparse matrix methods utilising REML.

The development of high-density single nucleotide polymorphism (SNP) arrays enabled researchers also to estimate heritabilities in a new way, utilising genetic relationship matrices in populations without pedigree data (132,146). This is called SNP-based heritability. It must be noted, however, that in the case of complex traits and disorders, a SNP-based heritability has limitations. The SNPs do not capture all the genetic variation in the genome (147), and often a gap is observed between the heritability estimates from SNP-based data and from pedigree-based or, in humans, also twin data. Such a gap can be observed in **Table 3** between the heritability estimates from pedigree and genomic data by Sánchez-Molano et al. (2014) (139). This is the case of 'missing heritability' that has provoked discussion and research for more than a decade. Some of the proposed explanations are: 1) a large number of common variants that are not detected due to their low effect size, 2) rare variants with large effects that, in addition to their rarity, may be missed due to stringent minor allele frequency cut-off thresholds during pre-GWAS quality control, and 3) structural variation (147). It has been postulated that the use of whole genome sequence (WGS) data could eradicate the missing heritability issue. However, as Young (2019) (148) noted, even with high-quality WGS data there are some methodological challenges, exacerbated especially in the case of rare variants, which need to be resolved before the problem of missing heritability becomes history.

The pedigree-based studies have resulted in other important findings. Genetic correlations between different phenotypes have been reported. Genetic correlation measures the genetic relationship between two phenotypes and therefore can reflect pleiotropy (149). In the context of canine hip dysplasia, genetic correlations have often been estimated between different hip dysplasia phenotypes. However, there are several estimates of genetic correlation between hip and elbow dysplasia in different breeds, which indicate that the two dysplasias do share some additive genetic effects. Some of these and other genetic correlations from different studies are presented in **Table 4**.

**Table 4.** ***Estimated genetic correlations from some genetic studies of hip dysplasia.***

| Estimated genetic correlation ($r^2$) (SE) | Phenotype pair | Breed(s) | Reference |
|---|---|---|---|
| 0.41 (0.09)[1], 0.42 (0.10)[2] | BVA hip score and untransformed ED score[1] or transformed ED score[2] | LR (UK) | Lewis et al. (2011) (150) |
| 0.12 (0.02) | OFA hip score and ED score | A multi-breed cohort including 74 breeds (US) | Hou et al. (2013) (26) |
| 0.67[1], 0.91[2], 0.84[3] | Nine different BVA hip traits | GS | Wilson et al. (2013) (151) # |
| 0.66 (0.13)[a], 0.51 (0.04)[b], 0.98 (0.02)[c], 0.39 (0.32)[d], 0.90 (0.11)[e], 0.50 (0.19)[f] | OFA hip score and ED score | a= GS<br>b= BMD<br>c= GD<br>d= SAM<br>e= LR<br>f= GR | Oberbauer et al. (2017) (141) * |
| -0.28[a], -0.21[b], -0.29[c] | DI and hip-extended score | a= GS (US, purpose-bred)<br>b= LR(US, purpose-bred)<br>c= GR (US, purpose-bred) | Leighton et al. (2019) (140) × |
| 0.30 (0.03)[1], 0.29 (0.03)[2], 0.14 (0.05)[3] | FCI hip score and ED score[1] or elbow arthritis[2] or FCP[3] | GS (DE) | Stock et al. (2011) (152) |
| 0.41 (0.09) | BVA hip score and transformed ED score | LR (UK) | Woolliams et al. (2011) (153) |
| 0.77 (0.04)[1], 0.98 (0.04)[2], -0.61 (0.05)[3], 0.06 (0.10)[4], 0.83 (0.07)[5], -0.75 (0.03)[6], -0.14 (0.09)[7], -0.64 (0.08)[8], -0.19 (0.15)[9], 0.09 (0.08)[10] | Comparisons 1.-10. ǂ | Four breeds combined (NL): LR, GR, BMD, NF | Lavrijsen et al. (2014) (154) ǂ |

Genetic correlations can have values between -1 and 1. × Leighton et al. (2019) reported genetic correlations obtained from two different methods and some quite extensive differences were observed between them (140). They undertook a thorough investigation to determine which estimates would more likely be correct, which led them to choose the Bayesian estimates and therefore only those are reported here. * Oberbauer et al. (2017) had heritability estimates from 60 different breeds in the US (141); the results for the breeds that overlapped with the target breeds of this thesis are listed here. ǂ Lavrijsen et al. (2014) reported numerous genetic correlations for different hip and elbow phenotypes (154). Only the genetic correlations between the different hip phenotypes and osteoarthritis of the elbow are presented here; 1. Hip osteoarthritis and hip congruity, 2. Hip osteoarthritis and shape of the acetabulum and femoral head, 3. Hip osteoarthritis and Norberg angle, 4. Hip osteoarthritis and elbow osteoarthritis, 5. Hip congruity and shape of the acetabulum and femoral head, 6. Hip congruity and Norberg angle, 7. Hip congruity and elbow osteoarthritis, 8. Shape of the acetabulum and femoral head and Norberg angle, 9. Shape of the acetabulum and femoral head and elbow osteoarthritis, 10. Norberg angle and elbow osteoarthritis. # Wilson et al. (2013) also reported a large number of genetic correlations for different BVA hip trait comparisons, but standard errors were not available (151). Only the following comparisons are presented here: 1. Norberg angle and femoral head remodelling, 2. Caudal acetabular edge and femoral head remodelling, and 3. Dorsal acetabular edge and femoral head remodelling. BVA= British Veterinary Association, OFA= Orthopedic Foundation for Animals, FCI= Fédération Cynologique

Internationale. ED= Elbow dysplasia, DI= Distraction index, FCP= Fragmented medial coronoid process of the ulna. LR= Labrador Retriever, GS= German Shepherd, GR= Golden Retriever, BMD= Bernese Mountain Dog, GD= Great Dane, SAM= Samoyed.

Another study, which used a quantitative genetics approach, revealed additive and dominance effects for different hip dysplasia related phenotypes (155). The study was conducted with an experimental pedigree (156) comprised of dysplastic Labrador Retrievers that were mated with non-dysplastic Greyhounds (a breed that is well-recognised for having a very low prevalence of hip dysplasia) and their progeny, including backcrosses (155). The researchers found that age at detection of epiphyseal ossification of the femoral head (OSS) had a significant additive genetic effect, and that Greyhound alleles decreased this phenotype by 3.6 days (standard error= 0.9) (155). Body weight at OSS had a significant additive genetic effect; an increase of 1 ounce (28.35 grams) in body weight delayed OSS by 0.20 days (standard error=0.03) (155). In addition, both the DI and DLS had an additive genetic effect. Greyhound alleles decreased the DI by 0.2 (standard error=0.04), whereas they increased the DLS by 12.5 ± 2.8% (155), meaning decreased laxity of the hip joint and increased coverage of the femoral head, respectively. Significant dominance effect was observed for DLS but not for DI or OSS. Greyhound alleles further increased the DLS score by 8.2 ± 3.0% on top of the abovementioned 12.5% increase due to additive effects (155).

The technological advancements during the genomics era have provided researchers with some powerful tools, although the more traditional methods, such as the pedigree-based linkage analysis (157), still serve us well in some cases. However, in the context of polygenic (complex) disorders, genomic tools, such as high-density SNP arrays and WGS techniques, are usually required. The first are needed for mapping the associated loci, which are potentially numerous (e.g. over 700 genes in a multitude of loci are known to influence human temperament) (158). More importantly, finding the causal variants behind the disorder usually necessitates the use of WGS techniques, because often the SNPs that demonstrate association in a genome-wide association study (GWAS) are not the causal ones. Moreover, due to the LD structure of the canine genome (10), the causal variants can be quite a long way from the associated loci.

The power of an association study to detect effect alleles depends largely on the sample size and also on the density of the SNP array used. For example, to detect a large-effect allele with the current SNP array that includes ~173000 markers, at least 100 cases and the same number of controls are needed (159). The denser SNP arrays, and especially WGS data, increase the statistical power. Hayward et al. (2016) demonstrated that, in comparison with the abovementioned current SNP array that has one SNP per 13 kb, an array with one SNP per 2 kb increased the power to detect the loci from less than 30% to 38% (12). This results from the more accurate tagging of the causal loci (12). However, sample size has more weight in relation to power than increasing the array density; doubling the sample size increased the power from less than 30% to ~ 50% in Hayward et al.'s simulation (2016) (12). They also highlighted that a within-breed GWAS has higher power at lower sample sizes than an across-breed study (12). The differences in power between across- and within-breed study designs result from the LD structure as LD breaks down slower within some breeds (12). However, it is often easier to collect a large across-breed study cohort than a corresponding number of dogs from one breed.

The first marker-based studies revealed quantitative trait loci (QTL) that were associated with the disorder. Chase et al. (2004), one of the first to carry out such an investigation, used about 500 microsatellites (also known as simple sequence repeats or short tandem repeats) in a cohort of Portuguese water dogs (160). The analysed phenotype was the Norberg angle. They identified two QTLs on CFA1, approximately 95 Mb away from each other (160). One QTL was associated with the Norberg angle of the left hip, while the other QTL was associated with the right hip measure (160). Both QTLs accounted for ~ 15% of the genotypic variation and for both QTLs heterozygous haplotypes associated with extreme phenotypes (160). Todhunter et al. (2005) used 240 microsatellites to map QTL in a cohort based on dogs from the abovementioned experimental pedigree (156). The phenotypes they analysed were DI, DLS and Norberg angle, and the principal components that were derived from these phenotypes (161). QTLs on twelve different chromosomes were revealed, of which eight harboured QTLs for more than one phenotype or principal component (161). The associations were significant at the chromosome-wide significance level of $P < 0.05$, or $P < 0.01$ in the case of the QTLs on CFA11 and -29 (161). Todhunter et al. (2005) observed both additive and dominance effects, and that some of the QTLs had alleles with protective effects, while others seemed to impact the phenotypes in a worsening manner (161). Marschall and Distl (2007) also utilised microsatellites in their cohort of German Shepherds (162). They had only half the number of markers but 1.5x more dogs in their data compared to (160), and they used hip dysplasia status derived from the FCI score as the phenotype (162). They revealed nine genome-wide significant QTLs on chromosomes 1, 3, 4, 8, 9, 16, 19, 26, and 33 (162).

Since the studies by Chase et al. (2004) (160), Todhunter et al. (2005) (161), and Marschall and Distl (2007) (162) paved the way, much effort has been put into mapping the loci to reveal the causal variants for canine hip dysplasia. Genetic studies of canine hip dysplasia from the last ~15 years, including the three described above, are summarised in **Table 5**.

**Table 5.** ***Genetic studies of canine hip dysplasia from the last fifteen years.***

| Reference | Type of the study and genetic data (N dogs/ N markers*) | Phenotypes | Breeds | Revealed loci or associated SNPs (bold if causal variant found) |
|---|---|---|---|---|
| Chase et al. (2004) (160) | QTL mapping[V] with STR (286 / ~ 500) | Noa | PWD | Two QTLs on CFA1. One for left and one for right hip Noa. |
| Todhunter et al. (2005) (161) | QTL mapping[R] with STR (152 / 240) | DI, DLS, Noa and their PCs | LR, GH and their crosses [ǂ] | QTLs on CFAs 4, 9, 10, 11, 16, 20, 22, 25, 29, 30, 35 and 37. |
| Marschall & Distl (2007) (162) | QTL mapping[L] with STR (459 / 261) | FCI hip score | GS | QTLs on CFAs 1, 3, 4, 8, 9, 16, 19, 26 and 33[G]. QTLs on nineteen CFAs[C]. |
| Liu et al. (2007) (163) | iQTL/QTL mapping[M] with microsatellites (148 / 240) | OSS | LR, GH and their crosses [ǂ] | QTLs on CFAs 1[i], 3, 5, 8[i], 9, 17, 22, 28[i]. |
| Zhu et al. (2008) (164) | QTL mapping[LB] of CFA11 and CFA29 with SNPs (257 / 282) | DI | LR, GH and their crosses [ǂ] and GS | One QTL on CFA11 and two on CFA29. |
| Phavaphutanon et al. (2009) (165) | QTL mapping[LB] with microsatellites (192 / 276) | DI, DLS, Noa and their PCs | LR | Six QTLs on CFAs 1, 2, 10, 20, 22 and 32. |
| Zhou et al. (2010) (166) | GWAS (721 / Illumina 22K SNP array and a custom SNP array with 3,500 SNPs) | Noa and OA | LR, GH and their crosses [ǂ], and GS, NF, GR, RW, BC, BMD | Four SNPs associated[C] with Noa on CFAs 3, 11 and 30. Two SNPs associated[C] with OA on CFAs 17 and 37. |
| Friedenberg et al. (2011) (167) | QTL mapping[LB] of CFA11 with SNPs (257 / 111) following the findings of (164), and investigation of *FBN2* (1,551). | DI, DLS, Noa and OA | LR, GH and their crosses [ǂ], and fourteen other breeds. | ***FBN2*** **deletion haplotype** associated with hip dysplasia. Changes in gene expression were observed. |
| Pfahler & Distl (2012) (168) | GWAS; case-control (174 / Illumina 173K high-density SNP array) | FCI hip score (and ED) | BMD | Two SNPs on CFA14 and one SNP on CFA37 demonstrated association[G]. |
| Lavrijsen et al. (2014) (169) | GWAS; case-control (78 / Illumina 22K SNP array) | FCI hip score | LR | Multiple SNPs on CFAs 1[S], 5[S], 8[G], 15[S], 20[S], 25[S] and 32[S] showed association. |
| Fels et al. (2014) (170) | Two-stage association study for specific regions from (162): 1. 190 / 37 SNPs, 2. 843 / 10 SNPs (subset from the 37) | FCI hip score | GS | Nine SNPs on CFAs 3, 9, 26, 33 and 34 demonstrated association. |
| Fels & Distl (2014) (171) | GWAS; case-control (192 in GWAS, 834 in validation cohort / Affymetrix 127K SNP array) | FCI hip score | GS | Three SNPs on CFAs 24, 26 and 34 demonstrated association[G]. |
| Bartolomé et al. (2015) (172) | GWAS as part of a study aiming to develop a genetic prognostic test for HD; case-control | FCI hip score | LR | 250 SNPs were claimed to associate with hip dysplasia in a relatively small cohort, which is highly unexpected based |

| | | | | |
|---|---|---|---|---|
| | (240 / Illumina 173K high-density SNP array) | | | on the evidence from several other studies; quality control before the GWAS seems inadequate and may explain the questionable number of associated SNPs. |
| Hayward et al. (2016) (12) | GWAS (921 / Illumina 173K high-density SNP array and additional 12,143 custom markers) | Noa | An across-breed cohort including 69 breeds, and crossbreeds. | One SNP on CFA28 demonstrated association[G]. |
| Fealey et al. (2017) (173) | GWAS (392 / Illumina 173K high-density SNP array and additional 12,143 custom markers) | PCs of pelvic morphology, Noa and AI | An across-breed cohort including 51 breeds, and crossbreeds. | Two SNPs on CFAs 15 and 16 demonstrated association[G] with two PCs, respectively. |
| Huang et al. (2017) (174) | GWAS (921 / Illumina 173K high-density SNP array and additional 12,143 custom markers). Same cohort as in (12) | Noa | An across-breed cohort including 69 breeds, and crossbreeds. | Three SNPs on CFAs 15, 28 and 36 demonstrated association[G]. |
| Todhunter et al. (2019) (175) | Gene expression analysis (RNA-seq); hip capsule or ligament of the femoral head from cases and controls. | DI, DLS and Noa[+] | LR and two different breed crosses. | A total of 131 genes were differentially expressed. Of these, over 20 had been previously linked with hip dysplasia, osteo- or rheumatoid arthritis phenotypes, or with altered skeletal development. |

*Number of markers if applicable. ǂDogs from an experimental pedigree, introduced in (156). [+]Cases and controls were determined by joint evaluation of at least two of the measured phenotypes. [V]Variance-based association test. [R]Regression interval mapping. [L]Linkage analysis. [M]Maximum likelihood-based method, [LB]Multipoint linkage analysis using Markov chain Monte Carlo. [G]Genome-wide significant, [C]Chromosome-wide significant, [S]Suggestive association with some given level of significance. iQTL= Imprinted QTL (163); chromosomes that are marked with [i] harboured iQTL. STR= Short tandem repeat/microsatellite, CFA= Chromosome for Canis familiaris. Noa= Norberg angle, DI= Distraction index, DLS= Dorsolateral subluxation score, OSS= Age at the onset of femoral head ossification, PC= Principal component, OA= Osteoarthritis, ED= Elbow dysplasia, HD= Hip dysplasia, AI= Angle of inclination between the femoral neck and diaphysis. FCI= Fédération Cynologique Internationale. PWD= Portuguese Water Dog, LR= Labrador Retriever, GH= Greyhound, GS= German Shepherd, NF= Newfoundland, GR= Golden Retriever, RW= Rottweiler, BC= Border Collie, BMD= Bernese Mountain Dog. FBN2= Fibrillin 2.

**Table 5** indicates that many loci on 28 different autosomes have been revealed to associate with canine hip dysplasia. Causal variants are still scarce, and for a long time Fibrillin 2 (*FBN2*) has been the only gene for which there is more evidence than an association. Even for *FBN2,* it took several years before the first findings could be corroborated as none of the association analyses after (167) demonstrated association between the studied phenotypes and the locus of this gene, as was noted, for example, by Lavrijsen et al. (2014) (169) and Hayward et al. (2016) (12). This may have resulted from genetic discrepancies between study populations, as Lavrijsen et al. (2014) remarked, but it could also have been the outcome of different phenotypes used in the studies, or a

combination of factors. Ultimately, Todhunter et al. (2019) observed a significantly increased expression of *FBN2* in the dysplastic joint capsule compared to non-dysplastic tissue. Moreover, the expression of a fibrillin interfacing protein named elastin microfibril interface 3 was also increased in the dysplastic capsule tissue. Todhunter et al. (2019) emphasised that it is unknown if these changes were a response to tissue damage or if they expedite laxity in hip joint capsules (175).

Clearly, certain chromosomes are represented more than others, although the loci within these chromosomes are not exactly the same between the studies. Association with different loci on CFA1 have been observed in five different studies (160,162,163,165,169), all of which were conducted in different cohorts and most with different phenotypes (**Table 5**). Three of the five studies indicated two different QTLs within CFA1 (160,163,165). Loci on chromosomes 3, 9, and 11 have been indicated in more than three different studies each (CFA3 in (162,163,166,170); CFA9 in (161–163,170); CFA11 in (161,164,166,167)). For the loci on CFA3 and CFA9, there is evidence from different study cohorts and different phenotypes (**Table 5**). All studies that indicated loci on CFA11 were executed in at least partially overlapping cohorts that used either DI, DLS and Norberg angle, or at least one of these phenotypes. Overall, the majority of the divergent findings between the studies are probably due to differences in study designs (i.e. in methods, phenotypes and study cohorts).

## 2.6 Osteoarthritis

Osteoarthritis is a progressive, debilitating condition that affects dogs and humans (24,176), among other species. However, the incidence and rate of osteoarthritis in dysplastic hip joint varies considerably between individuals (24,176). Chronic pain and impaired mobility induced by this disease are some of the major clinical symptoms that can notably decrease quality of life in human and canine patients (90,176,177). Osteoarthritis is not just a disease of the cartilage, it affects the whole joint (176,178), and although we concentrate on the synovial (hip) joint here, it can affect any type of joint. Ligament failure in both humans and dogs has been associated with the subsequent development of osteoarthritis in large joints (179,180), following from the increased joint instability (178,180,181). Osteoarthritis has been called a wear-and-tear disease, but more recently it has been recognised as a complex multifactorial disorder which is influenced by mechanical, inflammatory and metabolic factors (176,182). Lately, it has also been increasingly emphasised that osteoarthritis is a dynamic process rather than a passive degenerative disease (176,182).

In osteoarthritis, the structural integrity of hyaline articular cartilage, subchondral bone, joint capsule, synovium, periarticular muscles, and the ligaments of the joints are compromised (176,178,182). The focus has long been on the articular cartilage and its degradation, although it is still unclear how and where osteoarthritis actually begins within the joint (178). Indeed, there is evidence of structural changes on the ligaments of the joints in humans even when the surface of the articular cartilage is still normal (179). In dogs, Lust and Summers (1981) described early alterations in hip joints in asymptomatic stage of osteoarthritis (183). They observed mild synovitis, increased volume of synovial fluid and *ligamentum capitis ossis femoris*, and lesions on the articular cartilage (183). Furthermore,

they postulated that the synovitis and the changes in synovial fluid and ligament volumes may have preceded, or at least coincided, the degradation of articular cartilage (183). Recently, Ulfelder et al. (2019) observed with arthroscopy that the 36 inspected hips of 20 dysplastic cases had at least ligament oedema or early fraying of the *ligamentum capitis ossis femoris* (184). All dogs also had at least mild synovitis (184). These changes were observed even when the cartilage structures were normal. The cartilage lesions were most common at the insertion of the *ligamentum capitis ossis femoris* (16/36 had surface fibrillation), and second most common at the middle of the acetabulum, although these changes were mostly of lesser severity (184).

### 2.6.1 Pathogenesis of osteoarthritis in a synovial joint

All tissues within the joint are affected as described above, but the chain of events is not fully understood. However, it is known that the molecular composition and organisation of the ECM is affected before the articular cartilage surface starts to deteriorate (90,185). The pathogenic processes of osteoarthritis in a synovial joint is summarised in **Fig. 4**.

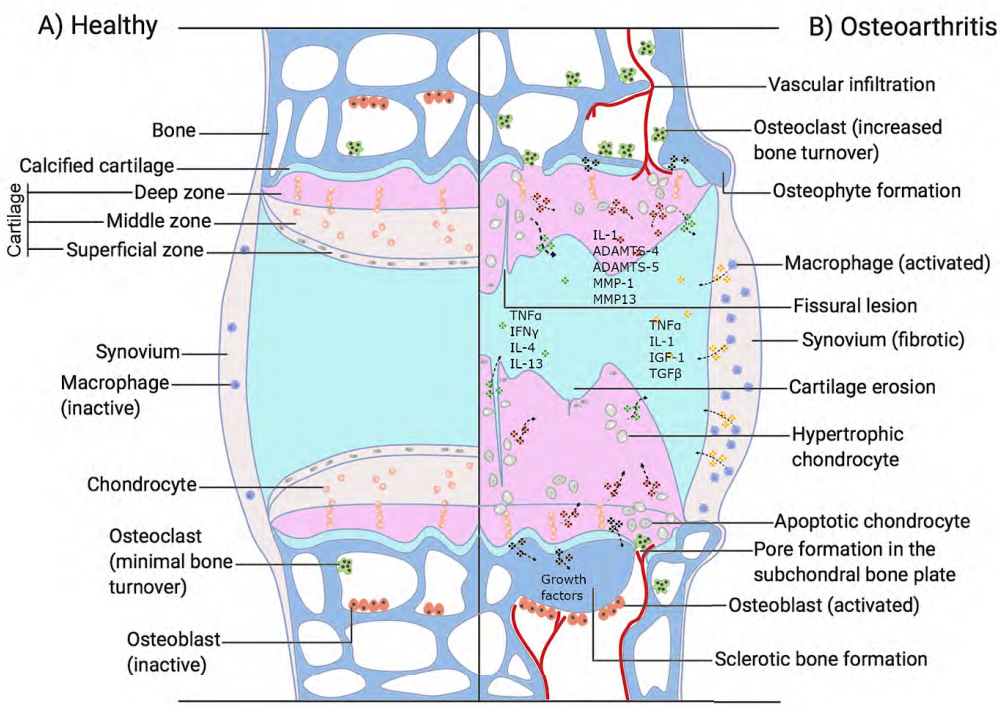


**Figure 4. Osteoarthritis in a synovial joint.** *Different signalling pathways and structural changes during the development of osteoarthritis. TNFα= Tumour necrosis factor alpha. IFNγ= Interferon gamma. IL-1, -4, and -13= Interleukin 1, 4 or 13. ADAMTS-4/-5= A disintegrin and metalloproteinase with thrombospondin motifs 4 or 5. IGF-1= Insulin-like growth factor 1. TGFβ= Transforming growth factor beta. MMP-1/-13= metalloproteinase 1 or -13* ***A)*** *Healthy synovial joint* ***B)*** *Synovial joint with osteoarthritis. Created with BioRender; modified from Hunter & Bierma-Zeinstra (2019)* (176).

When the articular cartilage is subjected to physical stimuli that leads to damage, the articular chondrocytes start to proliferate, turn hypertrophic and increase the ECM synthesis trying to initiate a repair process (90,185). These alterations increase the susceptibility of the articular cartilage to be disrupted by mechanical forces (90,176). This can lead to the destruction of cartilage integrity, while the proteoglycans are gradually lost and subsequently the collagen II is degraded (90,185). Moreover, the catabolic factors and pro-inflammatory mediators generated by the hypertrophic chondrocytes (**Fig. 4**) deregulate chondrocyte function and stimulate the synovium to respond with proliferative and pro-inflammatory processes (176). The proliferative synoviocytes release more pro-inflammatory products, which is followed by hypertrophy and increased vascularisation of the synovium (176). The catabolic changes also coincide with fibrillation of the articular

cartilage surface and production of fibrocartilage (185), which is not optimal cartilage type for synovial joints. Finally, the articular chondrocytes will undergo apoptosis and consequently the articular cartilage will be lost (90).

As the articular cartilage is not covering the joint surfaces any more, the bones come in contact, creating friction (90). The ongoing tissue injury and potential inflammation of the joint causes pain (90) via increased responsiveness of peripheral nociceptors (176). Mobility of the joint is also hampered (90). The bone turnover in the subchondral bone is increased and vascular invasion through the subchondral bone and the tidemark into the cartilage occurs (**Fig. 4**) (176). Subchondral bone marrow lesions and formation of osteophytes at the joint margins are other common features of progressing osteoarthritis (90,176,185).

### 2.6.2 The role of inflammation in osteoarthritis

Osteoarthritis has been classified as a non-inflammatory disorder for many years. It is only in the last decade that inflammation has become a well-recognised component in osteoarthritis, although Ehrlich suggested this in 1975 (186). Evidence is accumulating across species that inflammation contributes to the progression of osteoarthritis in several ways (187–196). Some of the main inflammatory mediators in osteoarthritis recognised today are different cytokines, nitric oxide, reactive oxygen species (ROS) and matrix degrading enzymes (191). Overall, there are several different pathways that may affect the inflammatory responses in osteoarthritis (187). These pathways and the molecules that belong to them interact strongly during the inflammatory responses in osteoarthritis.

There are a large number of inflammatory mediators and their antagonists that work interactively in osteoarthritis. Synovitis and infiltration of pro-inflammatory cytokines and chemokines into the synovial fluid are common signs of inflammatory responses in osteoarthritis (188,189,191). Furthermore, different types of white blood cells, such as macrophages, lymphocytes and leukocytes, have been detected to infiltrate the synovial fluid in osteoarthritis (192). In acute synovitis, neutrophils infiltrate the synovial fluid, while in more chronic synovitis, macrophages and lymphocytes are more common (196). Synovial macrophages are activated in osteoarthritis and produce pro-inflammatory cytokines and vascular endothelial growth factor (197). The latter promotes the release of different matrix metalloproteinases (MMPs), matrix degrading enzymes (198).

Pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin 1 (IL-1β) are also produced by the hypertrophic chondrocytes and synoviocytes. TNFα and IL-1β activate the expression of pro-inflammatory cytokines interleukin 6 and 8 from chondrocytes and synoviocytes (191,193). In turn, these cytokines add up to the inflammatory response because they activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB induces the production of catabolic factors such as matrix degrading enzymes (aggrecanases, also known as a disintegrin, and metalloproteinase with thrombospondin motifs ADAMTSs, and MMPs) (193), which will drive the inflammation further and increase cartilage degradation (187,191). MMP-13 and the aggrecanase ADAMTS5 have been identified as the key players in ECM degradation in osteoarthritis (187,191,199), although there are several other such factors with altered expression in osteoarthritis that should not be overlooked (199). Increased quantities of anti-

inflammatory cytokines interleukin 4, 10 and 13 have also been found in the synovial fluid of osteoarthritic joints (200). These molecules are produced by the synovial membrane and cartilage. They reduce the production of the pro-inflammatory cytokines and other inflammatory mediators like TNFα (200).

Most of the data in the studies mentioned above were from non-canine models or subjects. However, there are also studies that have demonstrated the role of inflammation in canine synovial joint osteoarthritis. Synovial inflammation and hypervascularisation of the synovial membrane in canine knee joints with surgically induced osteoarthritis was observed in a recent study by Korchi et al. (2019) (194). Dycus et al. (2013) studied the modulation of inflammation and oxidative stress in canine chondrocytes *in vitro* (190). They found that the canine chondrocytes responded to the oxidative stressors hydrogen peroxide, IL-1β or TNFα by decreasing the activity of superoxide dismutase (an enzyme that breaks down harmful superoxide radicals), among other things (190). From this, they concluded that exposure to such oxidative stress inducers could lead to disruption of chondrocyte and cartilage homeostasis that would subsequently promote the progression of osteoarthritis (190). Recently, Tellegen et al. (2019) made an intriguing observation on the relationship between osteoarthritis and the fibroblast growth factor 4 (*FGF4*) retrogene on CFA12 (195). *FGF4* retrogenes on CFA12 and CFA18 are known to be causative for canine chondrodystrophy via over-activation of fibroblast growth factor receptor 3 (201). This disorder has been associated with a decreased incidence of osteoarthritis and the findings from Tellegen et al. (2019) corroborated this for the *FGF4* retrogene on CFA12 (195). More severe synovitis and osteoarthritic changes were found in non-chondrodystrophic subjects in comparison with the chondrodystrophic counterparts (195). Tellegen et al. (2019) postulated that the non-chondrodystrophic cartilage may be more sensitive to pro-inflammatory stimuli in comparison with the chondrodystrophic cartilage, which may predispose these dogs to osteoarthritis (195).

In the past, obesity has been discussed as the main contributor to osteoarthritis due to the excessive and altered mechanical loading it poses on the joints (114,187,198). More recently, it has been suggested that obesity may contribute to the development and progression of osteoarthritis via disturbed lipid metabolism, low-grade inflammation and adipokines (198). For example, adipokines are known to contribute to the low-grade inflammation of obese human patients, which may influence cartilage homeostasis (187). Although the majority of adipokines are produced in white adipose tissue, chondrocytes can also produce different adipokines (187). They can then act on cartilage tissue degradation locally. Pro-inflammatory cytokines and adipokines interact, for example, IL-1β induces the production of some adipokines in chondrocytes (202). Moreover, the lipid metabolism of chondrocytes in osteoarthritis may be impaired. It has been observed that chondrocytes in osteoarthritis accumulate lipids, which is possibly due to decreased expression of genes involved in lipid efflux in affected articular cartilage (203). Although human and canine lipid metabolism are not exactly the same (204,205), it has been demonstrated that the lipid profile of the synovial fluid is highly similar between the species, both in the normal state and in osteoarthritis (205). Thus, similar lipid metabolism pathways or their parts could be expected to contribute to osteoarthritis in both species.

Above are some of the most well-recognised molecular factors of inflammatory responses in osteoarthritis. There is still much to learn, especially from the genetic

perspective. It must also be noted that a large proportion of the studies have been conducted on the knee joint tissues, which may not be perfectly equivalent to the environment in the hip joint and must therefore be accounted for in the interpretation. More studies are required to enlighten the inflammatory responses in osteoarthritis in canine hip joints.

### 2.6.3 Several genes and multiple molecular mechanisms are linked to pathogenesis of osteoarthritis

In addition to the inflammation-related pathways, there is cumulative evidence of different molecular mechanisms contributing to the pathogenesis of osteoarthritis, albeit most of it is not from canine models. Sandell (2012) (206) reviewed genetic studies of osteoarthritis and listed some genes that have been associated with it. Those that are associated with osteoarthritis in hip joints are listed in **Table 6**.

**Table 6.** ***Genes associated with osteoarthritis in the (human) hip and other joints. Adapted from Sandell (2014)*** (206)**.**

| Gene | Protein function | Tissue localisation | Pathway or system affected | Anatomical localisation |
|---|---|---|---|---|
| *ASPN* | TGFβ binding protein | Cartilage | Matrix development | Hip and knee |
| *BMP5* | Growth factor | Synovial joint | Joint development | Hip |
| *COL2A1, COL11A1, COL11A2* | Matrix components | All joint structures | Matrix development | All joints |
| *DIO2* | Deiodinase, regulates thyroid hormone | Growth plate | Development of hypertrophy | Widespread expression and present in hip |
| *DIO3* | Deiodinase, regulates thyroid hormone | Growth plate | Development of hypertrophy | Widespread expression and present in hip |
| *FRZB* | Wnt antagonist | Cartilage | Cartilage and bone development | Widespread expression and present in hip and knee |
| *GDF5* | Growth factor | Synovial joint | Joint development | Widespread expression and present in hip, knee and hand |

ASPN= Asporin, BMP5= Bone morphogenetic protein 5, COL2A1= Collagen type II alpha 1 chain, COL11A1= Collagen type XI alpha 1 chain, COL11A2= Collagen type XI alpha 2 chain, DIO2= Deiodinase 2, DIO3= Deiodinase 3, FRZB= Frizzled related protein, GDF5= Growth differentiation factor 5.

Sandell (2012) emphasised that the most prospective candidates belong in the growth and differentiation pathways that are essential for the formation and maintenance of synovial joints (206). Key players are transforming growth factor beta (TGFβ) superfamily, and Wnt signalling proteins. The TGFβ-signalling pathway is considered a major pathway in osteoarthritis (90,191). The TGFβ superfamily includes bone morphogenetic proteins (BMPs) and growth and differentiation factors, which participate in the foundation of the joint location but also induce the production or inhibit the formation of cartilage, bone and

other tissues in the joint (206). Wnt/β-catenin signalling participates in the regulation of arthritis development and progress (207). It has also been implicated because it is essential for cartilage and bone development, skeletal patterning and foetal development (90,175,206). Some factors from the Wnt signalling pathway, such as the frizzled related protein (*FRZB*) (**Table 6**) have been associated with osteoarthritis in the hip joint (206). *FRZB* has also been shown to increase loss of proteoglycans from cartilage in knockout mouse models. These mice were also more susceptible to chemically induced osteoarthritis (208). It was postulated that the cartilage damage was due to increased expression of MMPs in Wnt-dependent and Wnt-independent manner (208).

Interestingly, in a recent study, Todhunter et al. (2019) reported 131 differentially expressed genes in dysplastic hip joint capsule. Over 20 of these had previously been associated with hip dysplasia in humans or dogs, with osteoarthritis in hips or other joints, with rheumatoid arthritis, or with abnormal skeletal development (175). They found that the expression of an important component of the Wnt pathway, named Spondin 1, was increased in the joint capsule and ligament of the femoral head of dysplastic dogs and dogs with early osteoarthritis (175). Two other genes that encode for proteins linked to Wnt and TGFβ signalling were downregulated in dysplastic hip joint capsules (175). These were the dishevelled binding antagonist of beta catenin 2 and Wnt inhibitory factor 1 (175).

Todhunter et al. (2019) also demonstrated that hedgehog interacting protein (*HHIP*) expression was decreased in the dysplastic hip joint capsule in comparison to controls (175). The protein encoded by *HHIP* has been indicated to participate in human and induced murine osteoarthritis (209). Hedgehog signalling has been demonstrated to be activated during osteoarthritis, and in murine models, higher levels of hedgehog signalling in chondrocytes led to more severe osteoarthritis in Lin et al.'s study (2009) (209). However, Todhunter et al.'s (2019) (175) findings contradict Lin et al. (2009), who had reported that the expression levels of *HHIP/Hhip* were significantly increased in osteoarthritic articular cartilage in humans and mice (209), respectively. Nevertheless, these were different species and different tissues from different joints, which may impact the result. In addition, Todhunter et al. (2019) emphasised that it was uncertain if the decreased expression of *HHIP* was secondary to osteoarthritis or it actually contributed to the development of hip dysplasia in these dogs (175).

With regard to the dysplastic canine joint capsule, Todhunter et al. (2019) observed enrichment of genes involved in the ECM structure, epithelial to mesenchymal cell transition, myogenesis, growth factor signalling, cancer and immune pathways (175). Genes in retinoic signalling pathways and genes encoding ECM molecules (excluding proteoglycans) were enriched in the ligament of the femoral head in dysplastic hip joints (175). Many genes that have been implicated in human osteoarthritis and some that are related to injury response were differentially expressed in the dysplastic hip joint capsule (175).

There are also findings from other canine studies regarding the genetic background of osteoarthritis. The first study was by Chase et al. (2005) (210), at the beginning of the genomics era. They studied the radiographic changes that are related to the adverse remodelling of bone, alterations in joint morphology, and formation of osteophytes (210). One QTL on CFA3 associated significantly with osteoarthritis in their cohort of Portuguese Water Dogs (210). The QTL accounted for ~ 16% of the variation in osteoarthritis, involving

mainly cranial and caudal acetabular marginal osteophytes (210). In addition, it affected several other metrics of skeletal morphology, although with a different haplotype compared to the association with osteoarthritis. The QTL haplotype that associated with osteoarthritis was rare, and thus exhibited only a small contribution to the gene pool of Portuguese Water Dogs (210). Chase et al. (2005) concluded that the QTL possibly harbours a gene that may promote abnormal growth in joints. Their synteny analysis with mouse, rat and human genomes revealed two potential candidate genes in close proximity to the associated microsatellite: Insulin like growth factor 1 (*IGF-1*) and Desmuslin (210). Of these, *IGF-1* had been discussed as a potential factor in human osteoarthritis before this study was conducted (211). Later, Todhunter et al. (2019) observed a significant increase in the expression of *IGF-1* in joint capsules of dysplastic dogs (175). Thus, quite compelling evidence exists that this gene has an impact on osteoarthritis across species.

Burton-Wurster et al. (2005) (212) showed that MIG-6/gene 33 is upregulated in mechanically loaded canine articular cartilage and subsequently its expression was found to be increased in osteoarthritic canine cartilage (213). This gene responds to mechanical stress, among other stimuli, suggesting that the elevated expression level may result from continuous exposure to adverse biomechanical environments (213). It was also noted that MIG6/gene 33 may have an indirect molecular interaction with IGF-1 (213). Mateescu et al. (2008) found that chromosomes 5, 18, 23 and 31 harboured putative QTLs for hip joint osteoarthritis in dogs, measured as a necropsy score (214). In particular, the QTL on CFA18 had a marked effect on the necropsy score, and it was found syntenic to a locus on human chromosome 11 that has been linked to osteoarthritis (214,215). Zhou et al. (2010) found two different loci that revealed a chromosome-wide association with osteoarthritis in dogs (166). They suggested two proximal candidate genes: regenerating islet derived gamma 3 and par-3 partitioning defective 3 homolog B. The latter, as they discussed (166), had previously been associated with knee osteoarthritis in women (216).

The studies described above are only a small portion of the myriad of studies that have been published in this field. Many of the indicated molecular factors are linked to each other via different pathways. It has become increasingly evident that we need to capture these interaction networks to get the whole picture, in addition to the specific, and often small, effects a single molecule poses on osteoarthritis.

# 3 Aims of the study

Canine hip dysplasia is a common hereditary disorder with a complex genetic background. The genetic causes of this disorder have eluded researchers despite rigorous attempts to reveal them in multiple studies. Inadequate sample sizes and poor reproducibility have posed significant challenges in many of these studies. The resources provided to us by the canine DNA bank and the robust radiographic phenotypes from the FKC, along with the in-group veterinary experts, allowed us to aim for new genetic discoveries for canine hip dysplasia and osteoarthritis. The main aim of this thesis was to shed light on the genetic background of this disorder.

Specifically, the objectives in each study were:

i. To reveal the genetic loci that associate with the disease phenotype in case-control cohorts of German Shepherds with different FCI score categories. To find the causal variant(s) within the associated loci and to determine their functional effects *in vitro*. (**Study I**)
ii. To reveal the genetic loci that associate with the hip dysplasia phenotypes' FCI score, Norberg angle, FHCDAE, and osteoarthritis in a larger cohort of German Shepherds. (**Study II**)
iii. To validate the associated loci from studies I and II, and additional loci from eight other studies, in an independent cohort of 1607 dogs from ten different breeds, excluding German Shepherds. (**Study III**)

# 4 Materials and methods

## 4.1 Ethics statement

The dogs that participated in **studies I-III** were all privately owned pets. All owners had given written consent for their pets to participate. Ethical licences for collecting EDTA blood samples were approved by ELLA, the Animal Experiment Board in Finland, under the Regional State Administrative Agency of Southern Finland. Permit identifications for: **studies I-II** – ESAVI/7482/04.10.07/2015; **study III** – ESAVI/343/04.10.07/2016.

## 4.2 Study cohorts

**Study I** consisted of two association studies: the original association analysis and a meta-analysis with some additional samples. The cohorts in **Study I** consisted of 292 German Shepherds that participated in the original association study, and then an additional 233 dogs, a total of 525 German Shepherds in the meta-analysis (**Table 7**). Controls were dogs with FCI hip scores A/A (left hip/right hip) and mild cases were dogs with FCI hip scores C/C. The cases were also divided into two groups with a stringent (FCI hip scores D/D, D/E, E/D or E/E) or relaxed definition (FCI hip scores C/C, C/D, D/C, D/D, D/E, E/D or E/E). All dogs were born between 1993 and 2013. The cohort represented Finnish dogs registered by the Finnish Kennel Club, although some of them had been imported from other European countries (mainly Germany) and registered in Finland after importation. The German Shepherd population in Finland is tightly genetically linked to the German population as (breeding) dogs are commonly imported to Finland or Finnish bitches are mated with German studs.

The cohort in **Study II** comprised 775 German Shepherds before quality control (QC; see section 4.6.1 for details) (**Table 7**), including the dogs that were first used in **Study I**. Therefore, **Study II** was not independent from **Study I**. The dogs in this cohort were born between 1989 and 2016. In the case-control analyses, the controls were defined as dogs with FCI hip scores A/A and mild cases were dogs with FCI hip scores B/C, C/B or C/C. In **Study II**, the stringent case definition was the same as in **Study I,** but the relaxed case definition included dogs that had FCI hip scores B/C, C/B or C/C or worse on both hip joints.

The cohort in **Study III** was fully independent from **studies I** and **II**, consisting 1607 dogs from ten different breeds commonly affected by hip dysplasia but excluding German Shepherds (**Table 7**). The dogs in this cohort were born between 1990 and 2014. They were drawn from the canine DNA bank, which holds over 70,000 samples from more than 330 breeds. The DNA bank is maintained by Professor Lohi's canine genetics research group at the University of Helsinki.

Blood samples were collected from all dogs in **studies I-III** for genomic DNA extraction. The samples were ethylenediaminetetraacetic acid (EDTA) preserved blood and

were collected either at veterinary clinics or by trained research personnel. All samples were stored in the canine DNA bank.

**Table 7.** ***Study cohorts.***

| Study # | Breeds | Phenotypes | Number of dogs before QC (cases/controls/intermediate if applicable) | Number of dogs in analysis after QC (cases/controls) |
|---|---|---|---|---|
| I | GS | FCI hip score (case-control) | 531 (247/284) | 525 (247/278) |
| II | GS | FCI hip score (case-control), Norberg angle, FHCDAE, OA | 775 (322/356/97) | 769 (339/354/76)* |
| III | FL, GR, LAG, BMD, SAM, SWD, GD, LR, KBD, FH | FCI hip score (case-control) | 1607 (772/835) | 1570 (770/835) |

*The number of cases and intermediate phenotypes differ from the before quality control (QC) values on the left because before QC the phenotypes A/B, B/A, B/B, B/C or C/B were listed in the intermediate group. After QC, the dogs were analysed according to their worst FCI hip score, meaning that dogs with B/C or C/B were listed as cases in **Study II**. GS= German Shepherd, FL= Finnish Lapphund, GR= Golden Retriever, LAG= Lagotto Romagnolo, BMD= Bernese Mountain Dog, SAM= Samoyed, SWD= Spanish Water Dog, GD= Great Dane, LR= Labrador Retriever, KBD= Karelian Bear Dog, FH= Finnish Hound.

## 4.3 Phenotyping

FKC granted permission to use their FCI hip screening data and the hip joint screening radiographs in our research. All dogs in **studies I-III** had at least the FCI hip scores that were derived from the FKC database. In **Study II**, the hip screening radiographs of the participating dogs were loaned from the FKC, and two specialised veterinarians assessed the Norberg angle, FHCDAE and OA from them. Due to issues such as poor radiograph quality or poor positioning, 125 dogs had either missing radiographs or the veterinarians were otherwise unable to evaluate these phenotypes.

## 4.4 Reference sequences

For most parts of **studies I-III**, CanFam3.1 dog genome assembly was used as the reference genome. However, in **Study I**, a *de novo* German Shepherd reference sequence was designed and assembled based on scaffolds generated from resequencing data from one German Shepherd, which did not exhibit a deletion upstream of the gene *NOG*. This *de novo* reference was used in **Study I** in a dual luciferase reporter assay experiment.

## 4.5 Genotyping

### 4.5.1 DNA preparation

The laboratory personnel at the canine genetics research group at the University of Helsinki conducted the DNA extractions from the EDTA blood samples. They used a Chemagic Magnetic Separation Module I with a standard protocol by Chemagen (Chemagen Biopolymer-Technologie AG, Baeswieler, Germany). NanoDrop-1000 UV/Vis Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, US) was used for determination of DNA concentration from the samples. The samples were stored at -20℃.

### 4.5.2 Illumina high density 173K canine SNP array

The high-density canine SNP array from Illumina (San Diego, CA, US), which includes 173,662 SNPs, was used in **studies I** and **II**. Genotyping was carried out at Geneseek (Lincoln, NE, US) in several batches because the sample collection took place over several years. Batch effects were considered in the downstream analyses when required.

### 4.5.3 Agena MassARRAY® iPLEX SNP array

In **Study III**, 52 SNPs were genotyped with the MassARRAY® iPLEX system from Agena (Agena Bioscience GmbH, Hamburg, DE). The genotyping was executed in two separate batches by the Institute of Molecular Medicine Finland (FIMM) Technology Centre at the University of Helsinki. The first batch included samples from the following breeds: Labrador Retriever, Golden Retriever and Bernese Mountain Dog. The second batch contained samples from seven breeds: Spanish Water Dog, Karelian Bear Dog, Lagotto Romagnolo, Finnish Hound, Samoyed, Finnish Lapphund and Great Dane.

## 4.6 Pruning and analysis of the SNP data

### 4.6.1 Quality control

In **Study I**, the QC of the original SNP data from 293 dogs (original GWAS) was conducted using PLINK (v1.7) (217) and with GenABEL (version 1.7-6) (218) in R (version 3.5.0) (219). In PLINK, the cut-off for genotype missingness was 0.05. In GenABEL the SNP data was pruned with the following thresholds: minor allele frequency (MAF) was 0.05, sample-wise call rate was 0.90 and SNP-wise call rate was 0.95. The p-value cut-off for the HWE-check was < 0.00001. Tests for deviations from Hardy-Weinberg equilibrium (HWE) were done only in controls in **studies I-III** because affected individuals may show deviation from

HWE due to association with the disease (220). One sample was manually curated after the basic QC due to an outlier genotype.

The QC before the meta-analyses in **Study I** was completed in two steps. The first QC was done using PLINK (217) separately over the data sets before merging them. The thresholds described above were used and a strand flipping procedure had to be performed before the data merge. After merge, the final QC was carried out in GenABEL (218) over the whole data with the same QC thresholds. One dog had to be removed due to missing genotyping batch information.

The QC in **Study II** was executed similarly using PLINK (217) and GenABEL (218). Before merging the data from **Study I** and the new data, a preliminary QC was done over the genotyping batches. The same QC thresholds were used as in **Study I**, described above. In the final QC after the data merge, the same thresholds were used, except for the per sample call rate, which was 0.85.

In **Study III**, some of the SNPs and samples did not pass the initial QC of FIMM. The subsequent within- and across-breed data QC was carried out in PLINK (217). The breed-specific data were pruned with thresholds of: sample- and SNP-wise call rates were 0.90; MAF was 0.05; and p-value cut-off for HWE-check was $< 0.0001$. The QC for the across-breed data was executed in two steps. First, QC was conducted at breed level before merging the data. The QC thresholds were as described above for the breed-specified data but MAF limit was set to zero because some SNPs that may not pass it within a breed could pass it in the across-breed cohort. The second QC after the data merge was then conducted for the whole data with just a MAF threshold of 0.05.

### 4.6.2 Population structure and stratification control

Prominent population substructure was observed in **studies I** and **II**. The dogs were divided into clusters using a genomic relationship matrix in each study. First, the appropriate number of clusters were determined with Bayesian information criterion using an R-package named mclust (221). Then a covariate vector was created where each dog belonged to one of the clusters. These covariate vectors were utilised in **studies I** and **II** to manage any potential differences in disease association between the population sub-structures.

In **Study III**, a similar procedure was not possible due to the lack of genome-wide SNP data. Therefore, extra care was taken during the initial data collection; close relatives were avoided and only one dog from any core family was included.

### 4.6.3 Genome-wide association analyses

In **studies I** and **II**, the genome-wide association analyses were implemented with GenABEL (218) in R (219) utilising polygenic mixed models (222). In **Study I**, first the polygenic object was created with the GenABEL-function called polygenic, which maximises the likelihood of the given data under a polygenic model and creates a polygenic object for downstream use in association analyses (222,223). Then, two different association analysis methods were used: FASTA, a score test for association in related subjects (88),

and QTSCORE, which is a fast score test for association with genomic control (224,225). QTSCORE was used in parallel with FASTA because QTSCORE can be used to estimate the empirical genome-wide significance levels for the analysed SNPs, which is something that FASTA is incapable of performing.

In **Study II**, FASTA was used for all of the association analyses. Generalized linear model and linear model functions in R (219), named glm and lm respectively, were used for model fitting to assess the appropriate covariates for each analysed phenotype. The tested covariates were sex, age at radiographing, genetic cluster of the dog, genotyping batch, birth month and, for the Norberg angle, FHCDAE and OA, also the evaluator. The covariates that had a significant effect on the dependent variable (P-value < 0.05) were chosen to be used in the association analyses.

### 4.6.4 Assessment of linkage disequilibrium and estimation of the number of independently associated loci

In **studies I-III**, linkage disequilibrium (LD) between the associated SNPs was estimated to evaluate the number of independently associated loci in each study. In **studies I** and **II**, this was done by estimating the square of the correlation coefficient ($r^2$) (226) between the different markers with the r2fast-function (227) from the GenABEL-package (218). In **Study III**, estimation of LD (as $r^2$) for the 52 SNPs was carried out in PLINK (217).

#### *4.6.4.1 Estimation of the effective number of independent tests to determine the threshold for significance*

Due to the overly stringent nature of Bonferroni correction in genetic association studies (228), the effective number of independent tests was estimated to create a less conservative threshold for significance. SimpleM (229–231) was used for this task in **studies I** and **II**.

### 4.6.5 Association test in the validation study

In **Study III**, which was a validation study without genome-wide SNP data, the association tests were carried out in PLINK (217). The across-breed analysis was carried out with the Cochran-Mantel-Haenszel (CMH) test for 2x2xK stratified tables. The breed-specific analyses were performed either with an allelic $X^2$-test or with logistic regression. The analysis method that demonstrated better model fit in quantile-quantile plots of observed against expected P-values was chosen.

### 4.6.6 Analysis of candidate gene sets from Study III

We used search tool for the retrieval of interacting genes/proteins (STRING) version 11 (232) to assess if the 254 candidate genes from the validated loci (**Study III**) were enriched in any cellular pathways.

## 4.7 Targeted resequencing and fine-mapping of the associated loci on chromosome 9

During **Study I**, a targeted resequencing of a 7 Mb region on CFA9 was carried out to fine-map this region of interest. The procedure was executed by the DNA Sequencing and Genomics lab at the University of Helsinki. A total of 48 German Shepherds were included, 24 cases and 24 controls, which represented specific genotypes of seven SNPs located within this region. The libraries were paired-end sequenced with a MiSeq sequencer (Illumina, San Diego, CA, US). Raw reads were filtered using PRINSEQ (233) and after QC the reads were mapped to CanFam3.1 using the Burrows-Wheeler alignment tool (234). A tablet and an integrative genomics viewer were used to visualize the read alignments (235,236).

### 4.7.1 Variant analysis

In **Study I**, a pipeline to screen for coding variants from the resequencing data was implemented. A base quality check was completed using FASTX and then the reads were mapped to the reference genome (CanFam3.1) with the Burrows-Wheeler alignment tool (234). The variant calling was performed with the Genome Analysis Tool Kit version 3.5 (237) and SAMtools version 1.2 (238,239). ANNOVAR (240) was used to annotate the detected variants on Ensembl and NCBI gene annotation databases.

The statistical association between the phenotype and the SNP variants within the target area was assessed in 258 controls and 168 cases with moderate-to-severe hip dysplasia. To determine the independence of the variants and the phenotype, the Cochran-Mantel-Haenszel test variable $M^2$ was used. Finally, the statistical significance of the association of the SNPs with the phenotype was assessed using the null distribution of the maximum $M^2$, which was estimated with 10,000 permutations.

### 4.7.2 Fragment genotyping and analysis

In **Study I**, PCR was used to investigate a fragment of 400 bp, which encased a variant. Primers were designed with the NCBI Primer-BLAST tool (241). The primer sequences are located in the supplementary materials for **Study I (Study I/S11 Table)**. The primers were ordered from Oligomer (Helsinki, Finland) and annealing temperatures were calculated with the Thermo Fischer Scientific Tm calculator for Phusion DNA polymerase (242). A T100 Thermal Cycler (Bio-Rad, CA, US) was used to run the PCR with a standard 3-step protocol

for the Phusion high-fidelity DNA polymerase (Thermo Fischer Scientific, Waltham, MA, US). Standard 1.2% and 2% agarose (A9539, Sigma Aldrich, St. Louis, MO, US) gels with 1 x TBE buffer and ethidium bromide staining were used to run the PCR products. Gel imaging was done with an AlphaImager (Alpha Innotech, Kasendorf, Germany). The PCR amplicon was validated with sequencing at the DNA Sequencing and Genomics lab at the University of Helsinki. The PCR products that were ambiguous on gels were run through fragment analysis, which was also executed by the DNA Sequencing and Genomics lab at the University of Helsinki. Capillary electrophoresis was used to analyse the fragments. The size standard dye was GeneScan 500 ROX (4310361, Thermo Fischer Scientific, Waltham, MA, US). Peak Scanner v1.0 (Applied Biosystems, Foster City, CA, US) was used to analyse the fragment data.

## 4.8 Dual luciferase reporter assay

Three *NOG* enhancer sequence variant constructs were designed according to the findings of the targeted sequencing in **Study I**. Construct A was based on the *de novo* German Shepherd reference sequence that was assembled in **Study I**. Constructs B and C were different sized deletion variants from **Study I**. The construct sequences are located in the supplementary materials for **Study I (Study I/S7 Table)**. These *NOG* enhancer sequence variants were cloned into a pNL3.[*Nluc*/minP] NanoLuc luciferase vector from Promega (Madison, WI, US). A pGL4.54[luc2/TK] firefly luciferase from the same company was used as a control. HEK293 and U-2 OS cell lines were transfected with each plasmid and carrier DNA using Fugene HD transfection reagent from Promega (Madison, WI, US).

Luciferase activity was measured after 24 hours with the Nano-Glo Promega Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, US). The NanoLuc luminescence values were normalized against the control firefly luminescence. The data was analysed in R (219) with the Kruskal-Wallis rank sum test and subsequently with Dunn's test for multiple pairwise comparisons. Bonferroni adjustment for p-values was used and a p-value $< 0.05$ was considered significant.

# 5 Results

## 5.1 New protective and risk loci discovered for hip dysplasia in German Shepherds (Study I)

### 5.1.1 GWAS and subsequent meta-analyses reveal new protective and risk loci on chromosomes 1 and 9

The first GWAS was conducted with a relatively small cohort of German Shepherds with FCI hip scores: 132 cases with moderate-to-severe hip dysplasia and 160 controls with normal hips. The dogs were genotyped with the Illumina high-density 173K Canine SNP array (Illumina, San Diego, CA, US) and after QC, 92,315 SNPs remained for analysis. This original GWAS was run with GenABEL utilising polygenic mixed models and adjusting for population structure. We found a suggestive association on CFA9, but the analysis lacked power. Therefore, we genotyped 233 individuals more, after which we analysed these samples together with the original cohort in two meta-analyses. The first meta-analysis still entailed only the moderate-to-severe cases and increased number of controls ($N_{total}$ = 409); CFA9 was indicated again. The second meta-analysis compared mild to severe cases with all the controls ($N_{total}$ = 524) and it revealed loci on CFA1 and CFA9. None of the associations reached genome-wide significance and only the SNPs that passed an arbitrary threshold of 1.0x10-4 were included in **Study I/Tables 1-3**. Best power was observed in the first meta-analysis with P-values ranging from 7.6x10-5 to 2.2x10-6 (**Study I/Table 2**). The association on CFA9 in the first meta-analysis was over 45 times stronger than in any other loci in the genome.

Subsequently, we conducted LD analyses to investigate whether there was more than one locus within each chromosome. Indeed, two loci were implicated by $r^2$ for both chromosomes. This was corroborated with clumping analyses, a standard tool for estimating the number of independently associated loci. In the original GWAS, the first locus on CFA9 spanned from 30,993,502–32,382,532 bp and the second from 36,543,581–36,579,921 bp. The ORs for the SNPs in the first locus on CFA9 implied a protective effect, while the ORs for the second locus were between 2.25 and 4.90, indicating a risk effect (**Study I/Table 1**). The meta-analyses and subsequent LD analyses indicated four independent loci, which spanned 45,161,186–46,279,297 bp and 87,382,164–87,749,401 bp on CFA1, and 31,300,189–31,387,114 bp and 36,837,067–36,886,621 bp on CFA 9. The ORs for the loci on CFA1 were all < 1 (**Study I/Table 3**), indicating protective effects. The ORs in the first locus on CFA9 were all aligned with the results from the original GWAS. For the second locus, we observed opposing ORs but the LD between the opposing SNPs was moderate at best ($r^2$=0.68-0.71, **Study I/S1 Table**).

One of the associated SNPs on CFA1 resided within NADPH oxidase 3 (*NOX3*) and the rest of the SNPs within this locus were intergenic to NOX3 and AT-Rich interaction domain 1B (*ARID1B*) (**Study I/Table 4**). The SNPs on the other locus on CFA1 were intronic to either MAM domain containing 2 (*MAMDC2*) or protein prenyltransferase alpha subunit

repeat containing 1 (**Study I/Table 4**). The other top SNP on the first locus on CFA9 was intronic to ankyrin-repeat and fibronectin type III domain containing 1 (*ANKFN1*), while the other, located ~ 154 kb upstream from noggin (*NOG*) (**Study I/Table 4**). The SNP that represent the second locus on CFA9 resided between mitochondrial rRNA methyltransferase 1 (*MRM1*) and LIM homeobox 1 (*LHX1*) (**Study I/Table 4**).

### 5.1.2 Dogs with mild hip dysplasia are genotypically dissimilar to more severely affected dogs for the loci on chromosome 9

Next, we compared the genotype frequencies of the top SNPs between different phenotype groups to investigate if the loci behave differently in these scenarios: non-dysplastic controls (FCI hip score A/A) compared to bilateral mild hip dysplasia (FCI hip score C/C), or bilateral mild hip dysplasia compared to moderate-to-severe hip dysplasia (FCI hip score C/D, D/C or worse). We observed that dogs with mild hip dysplasia had significantly different allelic and genotypic frequencies for the top SNPs within the loci on CFA9 in comparison to dogs with moderate-to-severe hip dysplasia (**Study I/Table 5**). For the loci on CFA1, the differences between these groups were not significant. Moreover, the genotype and allelic frequencies between the mild dysplasia group and non-dysplastic controls were non-significant throughout the loci (**Study I/Table 5**). The dogs with mild hip dysplasia were differentiated from the moderate-to-severe cases by the homozygous risk genotype AG (**Study I/Table 6**) of two neighbouring SNPs in the first locus on CFA9.

### 5.1.3 Investigation of additional variants on chromosome 9

The two loci on CFA9 covered a region of approximately 5.3 Mb. We re-sequenced a region covering a total of 7 Mb (30,620,001–37,620,000 bp) in 24 cases and 24 controls to investigate if this region harboured any additional variants. We built a custom pipeline for systematic screening of the target region (see Methods in **Study I**). Originally, 30,197 variants in 21,140 positions were revealed, but after filtering, 61 remained for further examination (**Study I/S3 Table**). Of these, several variants for twelve genes were segregated between cases and controls with one of the following functional effects: downstream, nonsynonymous, exonic splicing, non-coding RNA exonic, splicing, upstream or UTR3 (**Study I/Table 7**). The genes were apoptosis antagonizing factor (*AATF*), *ANKFN1*, mediator complex subunit 13, myeloperoxidase (*MPO*), phosphatidylcholine transfer protein, phosphatidylinositol glycan anchor biosynthesis class W, RAD51 paralog C (*RAD51C*), ring finger protein 43 (*RNF43*), serine carboxypeptidase 1, Septin-4, testis expressed 14, and U1 small nuclear ribonucleoprotein.

Most of the coding variants were predicted to be tolerated and, for many non-coding variants, the evidence for functional elements such as chromatin marks was non-existent or low (**Study I/Table 7**). The genes *MPO*, *RAD51C* and *RNF43* harboured especially interesting variants. A potentially deleterious missense variant was found for *MPO* and it associated with cases. However, the variant was in such a position that it did not target the mature protein. For *RAD51C*, we found a potential regulatory variant and it was also

associated with cases (**Study I/Table 7**). Intronic variants near splicing regions were revealed for *RNF43*.

### 5.1.4 A regulatory variant found upstream of *NOG*

The re-sequenced region harboured novel SNPs that demonstrated significant association with the phenotype (**Study I/S5 Figure**). These SNPs concentrated on two loci corresponding to the loci that were previously indicated in our LD analyses. Visual inspection of pooled resequencing data revealed a 24-bp deletion variant at 31,453,837–31,453,860 bp that is within the first locus on CFA9. The location of the variant is depicted as a black vertical line on **S5 Figure** in **Study I**. The variant is located within a repetitive region close to *NOG*. Originally, the variant was observed in eight controls and one additional dog had a deletion of 27 bp. This finding was interesting because NOG and the sequence upstream from it are conserved across species (**Study I/S6 Figure**). In humans, this region entails a regulatory element that has binding sites for many transcription factors (243) (**Study I/Figure 4**). In addition, the region shows H3K4Me1 and H3K4Me3 histone mark peaks.

We then wanted to assess whether the variant existed in the rest of our cohort and if it associated with the phenotype. This was assessed with PCR; the fragment sizes were analysed on gel electrophoresis. Nine samples failed to give a product and one was ambiguous, which left us with 516 new genotypes. The deletion associated with non-dysplastic phenotype and mild cases but not with moderate-to-severe cases (**Study I/Table 8**). Moreover, the variant correlated with the SNP genotypes of BICF2P742007 and BICF2S23027935 in all phenotypes. We used logistic regression to estimate the significance of the protective effects of the deletion variant and GA SNP genotype. The protective effect of the deletion variant was strongest between mildly dysplastic dogs and moderate-to-severe cases (OR= 0.24, **Study I/Table 9**). Based on our model comparison (full model with the deletion variant or reduced model without it) and receiver operating characteristics curves, we determined that the full model better discriminated the controls from the moderate-to-severe cases, and the mild cases from the moderate-to-severe cases.

### 5.1.5 Reporter gene expression is downregulated *in vitro* by the *NOG* deletion variants

To investigate the effects of the deletion variants *in vitro*, we designed a luciferase reporter assay with three different sequence constructs, A, B and C. We used human embryonic kidney HEK293 and human osteosarcoma U-2 OS cell lines. Construct A had significantly higher luminescence compared to the other construct in the first experimental setup with HEK293 cells (**Study I/Figure 5**). In the setup with the U-2 OS cells, construct A had significantly higher luminescence than construct C (**Study I/Figure 5**). In all cases, comparison between control plasmid (pNL) and construct luminescence levels were significant.

### 5.1.6 Genome sequence for the *NOG* locus in dogs

The CanFam3.1 reference shows a gap within *NOG*. We used PCR and sequencing to close it. This enabled accurate positioning of the revealed deletion variant locus to the coding sequence of *NOG*.

## 5.2. A GWAS identifies three novel loci for canine hip dysplasia phenotypes and osteoarthritis (Study II)

**Study I** lacked power in the GWAS, so we wanted to collect a larger cohort and expand the study of hip dysplasia to other phenotypes. We also wanted to investigate osteoarthritis as a separate phenotype because it may have a partially distinct genetic background in relation to the other hip phenotypes (151,166,214). The GWAS was executed with GenABEL using polygenic mixed models. The appropriate covariates were estimated for each model (**Study II/Table 7**).

### 5.2.1 Two measures of joint incongruity map to chromosomes 9, 25 and 28

Joint incongruity is one contributing factor for hip dysplasia. The Norberg angle and FHCDAE can be used to assess hip incongruity and we used these phenotypes in **Study II**. Both phenotypes were evaluated for right and left hip joint, but we only used the worse measure in the association analysis. The Norberg angle demonstrated significant inter-observer variation in a linear regression model (P= 0.028, **Study II/Additional file 1**), in line with previous studies (44,244). Thus, we used the evaluator as one of the covariates in association analysis of the Norberg angle. These two phenotypes were highly negatively correlated (Pearson's r = -0.94, **Study II/Figure 1**), which was probably also reflected in the overlapping loci from the association analyses ($N_{total}$=642). However, associations were stronger for FHCDAE than for the Norberg angle.

One SNP on CFA9 and one on CFA28 demonstrated genome-wide significant association with FHCDAE but suggestive associations were also observed (**Study II/Table 1**). The genome-wide significant SNP on CFA9 was located at 31,477,907 bp; it previously showed suggestive association with hip dysplasia in **Study I** (**Study I/Table 1**). On CFA28, the genome-wide significant SNP was at 29,111,565 bp. None of the associations with the Norberg angle reached genome-wide significance but suggestive associations were observed on CFA9 and CFA25 (**Study II/Table 1**).

The SNPs that associated with FHCDAE on CFA9 were close to *NOG* and they were in high LD with each other (**Study II/Additional file 3**). These SNPs were shared between FHCDAE and the Norberg angle. One SNP with suggestive association to the Norberg angle was located upstream to *LHX1* on CFA9. On CFA28, both the SNP with significant association and the SNP with suggestive association to FHCDAE resided between the CDK2 associated cullin domain 1 (*CACUL1*) and nanos C2HC-type zinc finger 1 (*NANOS1*). The SNP with suggestive association with Norberg angle on CFA25 was intronic to solute carrier family 7 member 1 (*SLC7A1*).

### 5.2.2 Osteoarthritis maps to chromosome 1

We analysed osteoarthritis with a case–control GWAS. Controls (N=492) had no radiographic evidence of osteoarthritis and cases (N=163) had mild, moderate or severe osteoarthritic changes. Two SNPs on CFA demonstrated genome-wide significant association with osteoarthritis (**Study II/Table 2**). Suggestive associations were also observed on chromosomes 1, 9 and 25. The two genome-wide significant SNPs and four SNPs with suggestive association with the phenotype were between *NOX3* and *ARID1B*, previously reported in **Study I**. A total of six top SNPs spanned a region of over 1.1 Mb (at 45,161,186–46,279,297 bp) on CFA1 and still notable LD was observed between them ($r^2$= 0.63-1.00, **Study II/Additional file 3**). We considered them as one locus. The last two suggestive SNPs, 1.7 Mb away from the other six on CFA1, were intronic to transmembrane protein 181 and dynein light chain Tctex-type 1. The loci with suggestive associations with osteoarthritis on chromosomes 9 and 25 were between *MRM1* and *LHX1*, and intronic to *SLC7A1*, respectively.

### 5.2.3 Mild and moderate-to-severe hip dysplasia have different genetic backgrounds

We also carried out GWAS on different case-control categorisations of the FCI hip scores corresponding with **Study I**. This time dogs with FCI hip scores B/C or C/B were also included in the mild dysplasia group. The first comparison had 339 cases ranging from mild to severe and 354 controls, the second had 166 cases with moderate-to-severe hip dysplasia and 354 controls, and the third had 124 mild cases and 216 moderate-to-severe cases.

The same locus on CFA1 that showed genome-wide significant association with osteoarthritis was also genome-wide significant in the first comparison here (**Study II/Table 3**). None of the other comparisons resulted in genome-wide significant associations but two of the same SNPs on CFA9 demonstrated suggestive association in the second and third comparisons (**Study II/Table 3**). One of these SNPs was intronic to *AATF* and the other one located near this gene. Although these associations were only suggestive, the results of the last comparison support the observation made in **Study I** that mild and more severe hip dysplasia may have partially different genetic aetiology.

## 5.3. Validation of 46 genetic markers across breeds in canine hip dysplasia (Study III)

Years of rigorous research by different people has revealed numerous loci represented by different genetic markers, but to date their findings lack validation. We aimed to validate some of the markers that we found in **Study I** and also to validate some markers that have been reported to associate with hip dysplasia and osteoarthritis in other studies. The SNPs

that were unique to the more recent **Study II** were not included in **Study III** because at that time it had not been published.

**Study III** was carried out on a large cohort of dogs (N=1607) that comprised ten different breeds (**Table 1**). Fifty-two markers were selected, of which six were from our own **Study I**, ten were selected based on research by Wisdom Health, and the rest were from studies (139,166–172). Five of the markers from the Wisdom Health patent US10150998B2 (245) were also included in (169).

After quality control, 46 markers and 1570 dogs remained for the analysis. Dogs with a FCI hip score of A/A were controls (N=819) and dogs with a FCI hip score of C or worse on both joints were regarded as cases (N=751). A Cochran-Mantel-Haenszel 2x2xK test was used to analyse the across-breed data in PLINK 1.7 and the within-breed analyses were executed with either a $X^2$-test or with logistic regression.

### 5.3.1 Five markers on chromosomes 1, 14, 26, 33 and 37 associated with hip dysplasia across breeds

The across-breed analysis validated five SNPs on chromosomes 1, 14, 26, 33 and 37; they indicated significant association with the phenotype (**Study III/Table 1**). The SNP on CFA1 was from **Study I**. The SNP on CFA14 had originally associated with the FCI hip score in Bernese Mountain Dogs (168), while the marker on CFA26 had associated with the same phenotype in German Shepherds (171), as did the SNP on CFA33, but in a different study (170). Finally, the SNP on CFA37 had originally associated with osteoarthritis in a multi-breed cohort (166). The permuted P-values for the five validated markers varied between 0.049 and 6.1x10-3 (**Study III/Table 1**).

### 5.3.2 Several markers with protective or risk effects revealed in within-breed analyses

Twenty-two markers from thirteen different chromosomes were associated significantly with the phenotype (**Study III/Table 2**). There were differences between breeds in how many markers associated with the phenotype; for Bernese Mountain Dogs, none of the markers reached significance. Six markers were observed to be significant in more than one breed (**Study III/Table 2**). They were located on chromosomes 1, 11, 24, 26, 33 and 34. The markers on CFA11 and CFA33 were significant in three breeds each. Two marker pairs, one on CFA1 and the other on CFA8, were found to be in high LD so they were determined to represent two instead of four loci. Therefore, we finally validated 20 loci on 13 chromosomes within breeds. The ORs for the markers demonstrated risk and protective effects within seven breeds and only risk loci for two breeds (**Study III/Table 2**).

### 5.3.3 Neddylation-pathway was enriched in a STRING-analysis

The 21 loci on fourteen chromosomes embody a myriad of genes. We conducted two STRING-analyses with the web-based tool: 1. with 48 candidate genes from the loci that showed association with the phenotype across breeds; and 2. with 254 candidate genes from all the 21 significant loci. The first analysis did not reveal any enriched pathways, while the second showed enrichment of the neddylation-pathway (Reactome ID: CFA-8951664) with 12/220 genes in the gene set (false discovery rate 0.016) (**Study III/Figure 1**).

# 6 Discussion

In this thesis, novel loci were revealed and some previously reported ones were validated. Subsequently, new candidate genes for canine hip dysplasia and osteoarthritis were revealed. A putative regulatory variant was uncovered, and it was demonstrated to decrease reporter gene activity *in vitro*. Finally, a novel pathway for canine hip dysplasia was discovered. The studies were facilitated by active sample collection of hundreds of new samples and by the extensive resources of the canine DNA biobank at the University of Helsinki and the FKC database, as well as two specialised veterinarians who provided expertise in phenotyping. The study highlights the importance of fluent collaborations between clinical and basic research to advance the understanding of complex disorders. The results from these studies provide long-awaited new knowledge about the genetic background of canine hip dysplasia and osteoarthritis, two intertwined and complex genetic disorders.

## 6.1. New loci for canine hip dysplasia and a putative regulatory variant for an essential BMP inhibitor

Our robustly conducted association analyses uncovered four novel protective and risk loci on CFA1 and CFA9. The latter differentiated dogs with mild hip dysplasia from dogs with moderate-to-severe hip dysplasia. Deletion variants in a putative regulatory region of *NOG* demonstrated differential reporter gene activity *in vitro*. We also revealed novel candidate genes: *NOX3* and *ARID1B* on CFA1 and *RNF43*, *MPO* and *RAD51C* on CFA9.

*NOG* on CFA9 is a well-recognised inhibitor of the BMP family proteins. It is crucial for embryonic chondro- and osteogenesis, as well as joint formation (246–248). *NOG* missense mutations cause skeletal dysplasias in humans, which result from reduced secretion of the functional dimeric noggin protein that *NOG* encodes (249). However, *NOG* has not been associated with hip dysplasia before this study. In mice, overexpression of noggin impairs osteoblast function, which leads to osteopenia, microfractures and reduced rate of bone formation (246,250,251).

The deletion variants we discovered may have some regulatory effects on the expression of *NOG*, as the results from our luciferase reporter study suggest. We postulate that the German Shepherds susceptible to developing the more severe forms of hip dysplasia may exhibit suboptimal regulation of *NOG* activity. The CanFam3.1 reference sequence had a gap upstream of *NOG*, which hindered the accurate mapping of the discovered deletion variants in relation to the *NOG* exon. We therefore closed the gap, utilising PCR and sequencing, which relieved us from the positioning issue. The newly generated sequence may also be involved in the regulation of *NOG*. This suggestion is based on the high conservation degree with the corresponding human *NOG* promoter sequence and the vicinity of the region to *NOG*. However, canine *NOG* and its regulation require better characterisation before further conclusions can be drawn.

CFA1 harboured two loci that demonstrated association with canine hip dysplasia. The first spanned a region of 1.1 Mb and included the genes *NOX3* and *ARID1B*. *NOX3* is a non-

phagocytic member of the NADPH oxidase family that participates in the formation of superoxides and ROS. NADPH oxidase facilitates the production of hydrogen peroxide, which is used in a reaction cascade and has been shown to play a role in the initiation of articular cartilage degradation (252,253). *NOX3* is mainly expressed in the inner ear and foetal tissues (254), rendering its role in hip dysplasia uncertain. However, we observed an indirect link between the proteins NOX3, Rac family small GTPase 1 (RAC1) and trio Rho guanine nucleotide exchange factor (TRIO) in a STRING analysis (**Study I/S10 Table**). *TRIO* and *RAC1* have been implicated as candidate genes for canine hip dysplasia by Fels et al. (2014) (170).

*ARID1B* is a transcriptional regulator via chromatin remodelling (255). This gene is associated with a multisystemic Coffin-Siris syndrome, a rare hereditary condition that affects, among other things, skeletal system (256,257). A large proportion of Coffin-Siris patients (66%) exhibit joint laxity and consequently *ARID1B* is associated with it (256,257). We highlight, however, that dogs do not exhibit the multisystemic symptoms of the syndrome. On CFA1, there was one more possible candidate gene associated with the other locus on this chromosome. MAM domain containing 2 encodes a proteoglycan and it has been associated with elevated intraocular pressure (258).

Variants for *RAD51C*, *RNF43* and *MPO* were indicated in our variant analysis (**Study I/Table 7**). MPO and ROS have been implicated to play a role in the regulation of chronic inflammation (259–261). Elevated levels of MPO in the synovial fluid have been observed in humans with early osteoarthritis (253). Furthermore, MPO is one of the molecules participating in the reaction cascade leading to articular cartilage degradation (252,253), as mentioned above. Intriguingly, we revealed a missense variant for *MPO* that segregated with cases, although the mutated amino acid is not included in the mature protein. For *RNF43*, we found intronic variants near to splicing regions, which could be relevant. The protein encoded by this gene negatively regulates Wnt signalling (262), one of the key signalling pathways in osteoarthritis, as discussed in the literature review. The variant we found for *RAD51C*, a well-described recombination factor, may reside in a regulatory region but this requires further confirmation (263).

To conclude, utilising different genetic approaches we have discovered new loci for canine hip dysplasia and also found several novel candidate genes in the process. Moreover, we revealed several variants that may have an effect on the disease development and progression. Deletion variants of the gene *NOG* were demonstrated to downregulate reporter gene expression *in vitro*. These *NOG* variants were associated with normal and mildly dysplastic hip joints. Future studies should concentrate on investigating what effects the *NOG* variants or the other revealed variants may have in the canine hip joints and hip dysplasia.

## 6.2. GWAS of different hip dysplasia phenotypes and osteoarthritis reveal new loci on chromosomes 1, 9 and 28

The GWASs conducted in **Study II** resulted in remarkable progress by uncovering three novel genome-wide significant loci on chromosomes 1, 9 and 28 for a hip incongruity

phenotype and osteoarthritis. Two suggestive loci were also revealed on chromosomes 9 and 25. Moreover, the association with locus on CFA1 was shared between two binary traits: osteoarthritis and FCI hip score with a relaxed case definition analysed in case–control design. The study cohort in **Study II** partially overlapped the cohort in **Study I** and cannot, therefore, be regarded as an independent replication study.

The genome-wide significant locus on CFA1 for osteoarthritis and FCI hip score with relaxed case definition in this study (**Study II/tables 4 and 5**) was the same one (between *NOX3* and *ARID1B*) that was revealed in **Study I** in the second meta-analysis. In **Study I,** the same case definition was used but the cohort was smaller ($N_{studyI}$=524 and $N_{studyII}$=693). The association of the locus with osteoarthritis was 2.5 times stronger than with the FCI hip phenotype. We postulated that the overlap was probably caused by the fact that the FCI hip score is an aggregate trait (as explained in the literature review) and its evaluation includes the assessment of osteoarthritic changes on radiographs.

*NOX3* and the molecular functions of NADPH oxidase in a reaction cascade that lead to the initiation of articular cartilage degradation (252,253) were discussed in the previous section. Our STRING analysis (**Study II/Additional file 6**) revealed that NOX3 may interact with MMP2 and -9, which are matrix degrading enzymes that have been linked to canine hip dysplasia and osteoarthritis (264–266). *ARID1B,* the second candidate gene was discussed in the previous section.

We noted that our osteoarthritis loci did not overlap any of the previously described (166,210,214) loci that have associated with this phenotype. As previously noted in the literature review, the discrepancy may arise from methodological differences in the analysis, genetic heterogeneity between study cohorts, or from phenotyping methods in assessment of osteoarthritis. Incipient osteoarthritis is not visible in radiographs, so our findings probably reflect only the later stages of osteoarthritis, whereas, for example, the necropsy scores used by Mateescu et al. (2008) are based on the macroscopic evaluation of the osteoarthritic lesions straight from the joint.

In this study, the locus near *NOG* (discussed in detail in the previous section) associated with a phenotype that measures hip joint incongruity: FHCDAE (**Study II/tables 4 and 5**). The associations for the Norberg angle were non-significant, which is not surprising because the phenotype is highly affected by inter-observer variation (44,244), as noticed also in **Study II**. FHCDAE was not affected by similar bias. The association of this locus was 24 times stronger than for the FCI hip score phenotype in **Study I**. The possible impact of *NOG* on hip dysplasia was discussed in the previous section. However, we emphasise that *NOG* may be relevant to hip incongruity, while decreased noggin activity could potentially strengthen the bone in acetabulum through BMP signalling and help the repair of microfractures inflicted by adverse mechanical wear in growing dogs. Delayed ossification of the femoral head has been associated with later development of hip dysplasia (267,268) but the specific function of noggin in this process is unknown.

*CACUL1* and *NANOS1* are neighbouring genes near the CFA28 locus that reached genome-wide significance in association with FHCDAE. *NANOS1* upregulates metalloproteinase 14 (*MMP14*) (269), which is a powerful collagenolytic factor (270,271) that has been demonstrated to participate in synovial invasion via collagenolysis in rheumatoid arthritis (272). The *NANOS1–MMP14* interplay requires research into canine hip joint tissues. CFA28 has been associated with age at onset of femoral head ossification

(163). Liu et al. (2007) suggested that the CFA28 may harbour a maternally imprinted QTL for the age at onset of femoral head ossification (163). It has also been associated with the Norberg angle (12,174), more specifically with locus that is ~ 5.2 Mb away from the locus we found in **Study II**. The Norberg angle and FHCDAE are highly correlated, as previously noted, and this warrants studies to investigate if these two loci are related or not and if they harbour variants that may contribute to hip incongruity.

The suggestive associations on CFA9 and CFA25 revealed loci that harbour the genes *LHX1*, *AATF* and *SLC7A1*. *LHX1* was also observed in **Study I** and there is quite compelling evidence on its role in osteoarthritis. It has been demonstrated to be differentially methylated in osteoarthritis (273), and it is highly upregulated in osteoarthritis (274). Therefore, it remains an interesting candidate gene for canine hip dysplasia and it should be studied further. Conversely, *AATF* is located close to *LHX1*. Both these genes have been associated with macrophage inflammatory protein 1 beta (MIP-1 beta) levels (275,276); this is a cytokine that increases in synovial fluid during osteoarthritis (277). *AATF* resides ~ 803 kb away from the gene that encodes MIP-1 beta. Finally, SLC7A1 is an amino acid transporter, that transports cationic amino acids such as arginine across the plasma membrane (278). L-arginine may influence osteoarthritis through the nitric oxide pathway (279).

In conclusion, we have mapped several loci that harbour genes involved in different biological pathways. We stress that it is imperative to identify these pathways to understand the aetiology of canine hip dysplasia and osteoarthritis. Some genes may only have a circuitous effect to the phenotypes via other factors.

## 6.3. Validation study of 46 genetic markers within and across breeds

Over the last fifteen years, tens of loci for canine hip dysplasia have been revealed in several studies (**Table 5**), but replication studies have been scarce. We validated 21 loci on fourteen different chromosomes in **Study III**, and thus partially closed the replication gap. However, because our study did not include every single variant previously published, there is still work to be done in the future. Our replication analyses included 1570 dogs with FCI hip scores, and 46 markers. The validated loci harboured over 250 genes.

Five loci on chromosomes 1, 14, 26, 33 and 37 were validated across the analysed ten breeds. The locus on CFA1 was the *NOX3–ARID1B* locus we reported in **studies I** and **II**. The validated SNP resided closely upstream of *NOX3*. *NOX3* has been discussed in detail in the previous sections.

The locus on CFA14 originated from a study of Bernese Mountain Dogs (168). This locus did not associate with the phenotype in our cohort of this breed. We think this is possibly a consequence of the different case definitions between the studies. The associated marker in **Study III** is intronic to cortactin binding protein 2. Some recent phenotype associations from the GWAS catalogue (https://www.ebi.ac.uk/gwas/) are juvenile idiopathic arthritis, idiopathic osteonecrosis of the femoral head, and body mass index adjusted waist-hip ratio. The genomic region around the associated marker on CFA14 may be a susceptibility locus for obesity because body mass index adjusted waist-hip ratio was

mentioned for six of eight genes within a 1 Mb radius from the marker. Obesity, as discussed in the literature review, is a known risk factor for hip dysplasia and osteoarthritis via excessive biomechanical loading and via the inflammatory responses (198). Ultimately, in CFA14, Wnt family member 2 is only ~ 401 kb away from the associated marker. The role of Wnt signalling has also been discussed previously, but we want to highlight that genes in the Wnt/β-catenin signalling pathway may deserve some extra attention in future studies.

The marker on CFA26 was originally associated with hip dysplasia in German Shepherds. It is intronic to the kinase suppressor of ras 2 (*KSR2*). The KSR2 protein, which belongs to the Ras-Raf-MEK-ERK pathway, has been associated with obesity over species (280,281). Another candidate gene in this locus is nitric oxide synthase 1 (*NOS1*). Abnormal skeletal and skeletal muscle phenotypes have been reported for Nos1-mutant mice (282).

The marker on CFA33 was also originally associated with hip dysplasia in German Shepherds. It resides within PEST proteolytic signal containing nuclear protein (*PCNP*). This gene is expressed ubiquitously and may be involved in cell cycle regulation and/or genome stability pathway with its ubiquitination partner (283,284). ABI family member 3 binding protein (*ABI3BP*) is also located close to this locus. The protein binds collagen and glycosaminoglycans and thus, it is a component of the ECM. An intron variant of this gene was associated with joint hypermobility in humans (285).

Finally, the associated marker on CFA37 is intronic to neuropilin 2 (*NRP2*). This marker originated from an association study that used a multi-breed cohort including crossbreeds; it associated with osteoarthritis (166). In **Study III**, this marker did not associate with the FCI hip score in any particular breed but associated with the phenotype in the across-breed cohort. The multi-breed origin of the marker, as described above, may explain this. Furthermore, as mentioned before, FCI hip scoring includes the evaluation of osteoarthritis from the screening radiographs, which may explain the locus associated with this phenotype even though it associated with osteoarthritis in the original study. A similar observation was made in **Study II**. We suggest, based on the study of origin and our findings in **Study III**, that this could represent an 'across-breed locus' for canine hip dysplasia and osteoarthritis. In the original study, another gene in this locus was suggested as a candidate for osteoarthritis. We emphasise, however, that *NRP2*, which is closer to the associated marker than the gene Zhou et al. (2010) (166) suggested as the prime candidate, is also a good candidate because it is a receptor for vascular endothelial growth factors (VEGF) (286). There is a good amount of evidence of the functions of VEGF in osteoarthritis (286,287). It is not yet known how interaction between NRP2 and VEGF could impact osteoarthritis in dogs.

The within-breed analyses uncovered numerous loci. Our results also highlighted that breeds may have partially distinct genetic factors for complex disorders such as hip dysplasia and osteoarthritis. The number of associated loci varied between breeds and some markers also associated with hip dysplasia in more than one breed. The loci on CFA26 and CFA33 associated with hip dysplasia in more than one breed but also in the across-breed analysis. We stress that the results for Finnish Lapphund, Golden Retriever and Labrador Retriever may be inflated due to putative population stratification (**Study III/S2 Figure**). The ORs varied under and over 1 for the breed-specific loci, implying the existence of protective and risk loci. Some breeds had ORs over 5 for certain loci, which indicates quite strong association to the disease outcome (**Study II/Table 2**). Such high ORs have not been

very common in studies of canine hip dysplasia. Future studies should concentrate on such loci, especially in breeds that do not demonstrate marked inflation, such as Great Danes.

Because the loci revealed in the analyses in **Study III** were numerous, we decided to investigate whether any pathways are enriched in the candidate gene set (254 genes from 21 loci on fourteen chromosomes). Our STRING-analysis uncovered one enriched pathway: neddylation. Neddylation is an ubiquitination-like post-translational protein modification process that is conserved across species (288). Neddylation is crucial for cell cycle progression (289,290) and has been recently linked to inflammatory arthritis through increased activation of NF-κB (291). Neddylation also regulates T-cell and macrophage function in inflammatory response (292,293). As noted in the literature review, inflammation has an impact on osteoarthritis and therefore the role of neddylation-pathway in osteoarthritis should be studied.

To conclude, we validated 21 loci on fourteen chromosomes in **Study III**. We also found neddylation-pathway to be enriched in the candidate gene set and represent it for the first time as a candidate pathway for canine hip dysplasia and osteoarthritis. Our results highlight the complex genetic background of these disorders. Identification of causal pathways, genes and variants is an important task in the future so we can gain knowledge on the molecular aetiology of canine hip dysplasia and osteoarthritis and strive for better diagnostic tools and treatment options.

# 7 Concluding remarks

This doctoral dissertation unravelled new loci and validated many old ones, and also uncovered variants and a pathway that may affect the development of hip dysplasia and osteoarthritis in dogs. Because the disorder is similar in dogs and humans, dogs have been proposed to be a natural disease model for human hip dysplasia. Thus, better understanding of the genetic background in dogs may aid better treatment options in both species when the affected molecular pathways are well-described and their impact on the disease is understood.

Clearly, finding associated loci requires robust phenotypes and a large enough cohort. Moreover, to uncover the causal variants, high-quality sequence data is required. While WGS data is becoming more common, it is still relatively expensive, especially if deep coverage is required from many individuals. Imputation has been shown to improve the power of genome-wide association studies (14). Imputation could therefore be a good solution to help the studies of complex diseases as long as whole genome sequencing is not affordable in large cohorts. It must be noted, however, that imputation also requires good quality reference (WGS) data to cover as much of the variation in the target population as possible. In addition, consideration should be given to what could be good phenotypes for such studies. Accurate and repeatable phenotyping that describes the disease well is imperative.

The most efficient way to control this disease in dogs is selective breeding against it so that the prevalence of hip dysplasia is reduced in the future generations. Phenotypic selection is inefficient in the case of complex disorders because the phenotype of the dog does not reveal if it carries a risk genotype. EBVs, which are based on the phenotypes and pedigree data, have been observed to enable more efficient selection and have been successfully implemented for hip dysplasia in some populations. EBVs should be taken to use in all populations where their generation is feasible. Genomic selection that utilises EBVs and genotypic data is commonplace in livestock production. Compared to the traditional pedigree-based estimation method, the genomic EBVs can increase the accuracy of the predicted breeding values. This could further improve selection against hip dysplasia in dogs. However, genomic selection uses randomly chosen genetic markers across the genome. Choosing the markers based on association with the disorder does not improve accuracy and can lead to an upward bias, as is shown in (294). Therefore, association studies in this sense cannot improve genetic selection, which is important to note as a limitation for their application.

Therefore, the value of the genetic studies of canine hip dysplasia and other such complex hereditary disorders comes from the understanding of the biological processes and their potential application to treatment strategies. One more aspect that has not been studied to a great extent is the gene-environment interaction, as its role in the development of hip dysplasia is not well understood. Grasping these interactions could aid the management efforts of this disorder.

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